5.7 Agarose Gel Electrophoresis

Principle:	separation of DNA molecules according to their size and conformation in an electrical field across an agarose gel
Time	
Sampling:	-
Determination:	-
Sample material and quantity:	DNA, 2 ug
Results expressed in:	-
Equipment:	camera: Polaroid MP-4 Land Camera, film: Polaroid Typ 665, Polaroid GmbH, D-6050 Offenbach/Main 4 filter: Kodak Wratten 23A electrophoresis apparatus: model H4, Bethesda Research Laboratories GmbH, D-6078 Neu-Isenburg power supply: model 2103, LKB Instrument GmbH, D-8032 Gräfelfing centrifuge: model Sigma 2MK, Sigma Laborzentrifugen GmbH, D-3360 Osterode test tubes: type "Eppendorf", polypropylene, no. 3810, Netheler und Hinz GmbH, D-2000 Hamburg 65 magnetic stirrer: Ika Combimag RET, Janke und Kunkel GmbH und Co. KG, D-7813 Staufen UV lamp: Camag Reprostar/Transil-luminator, no. 29316, Camag, D-1000 Berlin 41

Chemicals:

sodium acetate, no. 6267,
bromophenol blue (BPB), no. 8122,
glycerol, no. 4093,
EDTA, no. 8418,
acetic acid, no. 63,
hydrochloric acid, no. 317,
ethidium bromide, no. 11615,
E. Merck,
D-6100 Darmstadt 1

tris(hydroxymethyl)aminomethane
(Tris), no. T 1503,
Sigma Chemie GmbH,
D-8024 Deisenhofen

xylene cyanol (XC), no. 1840-502,
LKB Instrument GmbH,
D-8032 Gräfelfing

agarose, no. 5510 UA,
Bethesda Research
Laboratories GmbH,
D-6078 Neu-Isenburg

sodium dodecylsulfate (SDS), no. 20760,
Serva Feinbiochemica GmbH und Co. KG,
D-6900 Heidelberg 1

ethidium bromide solution: 5 mg/l

Tris-acetate buffer (10 x):

Tris	400 mmol/l (48.4 g/l)
sodium acetate	200 mmol/l (27.2 g/l)
EDTA	10 mmol/l (3.72 g/l)

adjusted to pH 8.0 with acetic acid

loading buffer:

glycerol	500 ml/l
EDTA, pH 8.0	25 mmol/l
XC	1 g/l
BPB	1 g/l
SDS	1 g/l

gel composition:

agarose	2 g
Tris-acetate buffer (10 x)	20 ml
bidistilled water	180 ml

Procedure:

preparation of an agarose gel (10 g/l),
electrophoresis in Tris-acetate buffer

electrophoresis conditions:
Voltage: 40 V
time period: 16 h approx.

current: approx. 30 mA

gel stained for 5 min in 1 l ethidium bromide solution

photographs taken using transmitted UV light (300 nm)

Scientific version: SOP BC 237/3
Text version: 22.Jan.87

5.8 Growth of E. Coli

Principle: growth of E. coli under optimal conditions and amplification of plasmid DNA by addition of chloramphenicol

Time: -

Sample material and quantity: strain of E. coli containing plasmid

Equipment: shaking waterbath: WT,
Infors AG,
CH-4015 Basel

centrifuge: RC-5B,
rotor: type GSA,
Du Pont Instruments,
D-6350 Bad Nauheim

centrifuge tubes:
250 ml polycarbonate with screw tops, no. 03939,
Du Pont Instruments,
D-6350 Bad Nauheim

Erlenmeyer culture flasks:
250 ml and 1000 ml,
Faust GmbH,
D-5000 Köln 90

Chemicals: sodium chloride, no. 6404,
ethanol, no. 983,
E. Merck,
D-6100 Darmstadt 1

yeast extract, no. L21,
tryptone, no. L42,
Oxoid Deutschland GmbH,
D-4230 Wesel

ampicillin, no. A-9518,
chloramphenicol, no. C-0378,
Sigma GmbH,
D-8024 Deisenhofen

LB medium:

yeast extract	5 g/l
tryptone	10 g/l
sodium chloride	5 g/l

chloramphenicol solution:
34 g/l in ethanol

ampicillin: 100 mg/l medium

Procedure:

pre-culture:
inoculation from a petri dish or glycerol culture with a wire loop, incubation overnight (ca. 16 h) in a shaking waterbath at 37 degrees centigrade

main culture:
inoculation with 1 ml preculture/l, incubation at 37 degrees centigrade, determination of the adsorbance at 540 nm with a spectrophotometer every 10 to 60 min, addition of 1/20 volume chloramphenicol solution at an absorbance of 1.0, further incubation overnight, rapid cooling of culture on ice, pelletting of cells by centrifugation at 10000 rpm (16318 x g = 160080 m/s2) at 4 degrees centigrade for 30 min

Scientific version: SOP BC 274/1
Text version: 22.Jan.87

5.9 Plasmid DNA Preparation

Principle:	separation of supercoiled plasmid DNA from chromosomal and nicked plasmid DNA by banding in a density gradient (ethidium bromide-cesium chloride) during centrifugation
Time:	-
Sample material and quantity:	cells from 1 to 2 l of culture grown according to SOP 274/1
Results expressed in:	-
Equipment:	centrifuge: RC-5B, rotor: type GSA, OTD-65, rotor: type A641, Du Pont Instruments, D-6350 Bad Nauheim centrifuge tubes: 250 ml polycarbonate with screw tops, no. 03939, 50 ml polyallomer, no. 03140, Du Pont Instruments, D-6350 Bad Nauheim dialysis tubing: size: 1 cm, Medicell International Ltd., via Faust GmbH, D-5000 Köln 90
Chemicals:	sucrose, no. 7653, EDTA, no. 8418, n-butanol, no. 1990, E. Merck, D-6100 Darmstadt 1 tris(hydroxymethyl)aminomethane (Tris), no. T 1503, lysozyme, no. L-6876, caesium chloride, no. C-3011, Sigma Chemie GmbH, D-8024 Deisenhofen Triton X-100, no. 37240, ethidium bromide, no. 21238, Serva Feinbiochemica GmbH und Co. KG, D-6900 Heidelberg 1

1 mol/l Tris-HCl buffer:
121 g/l Tris, pH adjusted to 8.0 with HCl

Tris-HCl buffer 50 mmol/l Tris, 10 mmol/l EDTA:
6.1 g Tris and 3.7 g EDTA/l distilled water, pH adjusted to 8.0 with HCl

10 0/0 sucrose solution: 100 g/l sucrose in Tris-EDTA buffer (autoclaved before use)

25 0/0 sucrose solution: 250 g/l sucrose in Tris-EDTA buffer (autoclaved before use)

10 0/0 Triton X-100 solution: 100 ml Triton X-100/l

0.5 mol/l EDTA solution: 186 g/l, pH adjusted to 8.0 with NaOH

Triton lysis mixture:
Triton solution 4 ml
EDTA solution 50 ml
Tris buffer 20 ml
to 500 ml with distilled water

lysozyme solution:
10 mg/ml in Tris/EDTA buffer (made up fresh daily)

TE buffer:
10 ml Tris buffer,
2 ml EDTA solution,
988 ml distilled water

Ethidium bromide solution:
10 g/l

Procedure:

all steps carried out on ice. Cell pellet washed with 10 0/0 sucrose, resuspended in 25 0/0 sucrose, lysed with lysozyme, followed by EDTA and Triton lysis mixture, centrifuged for 1 h at 897625 m/s2 (91501 x g = 30000 rpm) at 4 degrees centigrade. 1 g cesium chloride/ml added to supernatant, then 1/20 volume ethidium bromide solution. Solution centrifuged at 14411 m/s2 (1469 x g = 3000 rpm) for 15 min to remove debris, filtered through cotton wool and centrifuged for 3 to 5 days at 748022 m/s2 (76251 x g = 25000 rpm) at 20 degrees centigrade.

	2 DNA bands visible in UV light (254 nm). Lower band removed with a syringe, ethidium bromide extracted with n-butanol, DNA solution dialysed 3 times against 1 l TE buffer each
Scientific version:	SOP BC 275/1
Text version:	22.Jan.87

5.10 Digestion of DNA

Principle:	digestion of DNA with restriction endonucleases at defined recognition sequences creating fragments with identical ends
Sample material and quantity:	any DNA
Time:	-
Results expressed in:	-
Equipment:	shaking waterbath: WTR-1, Infors AG, CH-4051 Basel, Schweiz centrifuge: type "Eppendorf", no. 5412, tubes: type "Eppendorf", no. 3810, Netheler und Hinz GmbH, D-2000 Hamburg 65
Chemicals:	DNA, plasmid pBR322, no. 481238, dithiothreitol (DTT), no. 197777, Boehringer Mannheim GmbH, D-6800 Mannheim 31 sodium chloride, no. 6404, magnesium chloride, no. 5833, E. Merck, D-6100 Darmstadt 1 tris(hydroxymethyl)-aminomethane (Tris), no. T 1503, Sigma Chemie GmbH, D-8024 Deisenhofen

Triton X-100, no. 37240,
Serva Feinbiochemika GmbH und Co. KG,
D-6900 Heidelberg 1

reaction buffers:
buffers made up 10 times concentrated, reaction conditions according to enzyme suppliers. Buffers sterile filtered and frozen in portions (100 ul)
at -20 degrees centigrade

Procedure: according to Maniatis et al. (1982)

Scientific version: SOP BC 238/1
Text version: 22.Jan.87

5.11 Purification of a DNA Fragment from an Agarose Gel

Principle: following agarose gel electrophoresis, removal of a piece of agarose containing the desired DNA fragment from the gel, extraction and purification of the fragment from the agarose

Time: following digestion with restriction endonucleases and separation of fragments by agarose gel electrophoresis

Results expressed in: -

Equipment: 5 ml plastic disposable syringe,
type Omnifix, no. 461605/7,
syringe needle, 0.6 x 26 mm,
type Erosa, no. 16,
Faust GmbH,
D-5000 Köln 90

NACS-PREPAC (TM),
mini-column, no. 550-1526 NP,
Gibco-BRL GmbH,
D-7514 Eggenstein

Chemicals:	tris(hydroxmethyl)aminomethane (Tris), no. T 1503, Sigma Chemie GmbH, D-8024 Deisenhofen EDTA, no. 8418, sodium chloride (NaCl), no. 6404, E. Merck, D-6100 Darmstadt 1 TE buffer: Tris 10 mmol/l EDTA 1 mmol/l 1.2 g Tris and 0.37 g EDTA/l distilled water, pH adjusted to 7.4 with HCl buffer A: NaCl in TE buffer 0.2 mol/l (11.7 g/l) buffer B: NaCl in TE buffer 1 mol/l (58.5 g/l) buffer C: NaCl in TE buffer 0.5 mol/l (29.2 g/l) buffer D: NaCl in TE buffer 2 mol/l (117 g/l)
Procedure:	solubilisation of agarose containing the DNA fragment by repeated freezing and thawing following passage through a syringe needle. Purification of the DNA by chromatography on a NACS-PREPAC mini column according to the manufacturer's instructions
Scientific version: Text version:	SOP BC 278/1 22.Jan.87

5.12 Radioactive Labeling of DNA 3'-Ends

Principle:	enzyme-directed incorporation of (alpha-35S) (for pUR222) - or (alpha-32P) (for PAL10)-dATP in 3'-DNA termini with DNA polymerase I (large fragment)
Time:	after digestion with appropriate restriction enzymes
Sample material and quantity:	up to 200 ug DNA with recessed 3'-ends from restriction enzyme cleavage

Results expressed in: -

Equipment:

freezer: no. 8218,
Forma Scientific,
via Labotect,
D-3406 Bovenden-Göttingen

incubator: Techne Dri Block DB-3,
Sabora, Karlheinz Bässler,
D-6395 Weilrod-Winden

test tubes: type "Eppendorf",
polypropylene, no. 3810,
centrifuge: type "Eppendorf",
no. 5412,
Netheler und Hinz GmbH,
D-2000 Hamburg 65

Chemicals:

dATP, no. D 6500,
Sigma Chemie GmbH,
D-8024 Deisenhofen

deoxyadenosine 5'-(alpha-35S)-
triphosphate, no. SJ 1304,
deoxyadenosine 5'-(alpha-32P)-
triphosphate, no. PB.10204,
Amersham Buchler GmbH und Co. KG,
D-3300 Braunschweig

DNA polymerase I (Klenow-enzyme),
no. 104532,
Boehringer Mannheim GmbH,
D-6800 Mannheim 31

Procedure:

according to Maniatis et al. (1982)

storage until determination:
unlabeled DNA at 4 degrees centi-
grade, labeled DNA at -75 degrees
centigrade

final concentration of components
in assay mixture:

DNA	200 mg/l
5'-(alpha-35S)- or 5'-(alpha-32P)-dATP	1 Ci/l
DNA polymerase I (large fragment)	125 KU/l

total assay volume: 20 ul to 1 ml

incubation: 10 min at RT

for chase reaction addition of 0.5 mol dATP/l

incubation: 5 min at RT

reaction stopped by heating for 10 min at 70 degrees centigrade

removal of the unincorporated 5'-(alpha-35S)- or 5'-(alpha-32P)-dATP with Sephadex G50 column (SOP BC 280)

Scientific version: SOP BC 245/3
Text version: 2.Dec.86

5.13 Base-Specific DNA Cleavage

Principle: DNA modification by specific chemical reactions to produce apurinic or apyrimidinic sites and strand breaks after alkaline heating

Time

Sampling: -

Determination: after DNA labeling, within 1 half-life of the radioisotope used for labeling

Sample material and quantity: radioactively labeled DNA, 1 ug

Results expressed in: -

Equipment: incubator: Techne Dri Block DB-3, Labora, Karlheinz Bässler, D-6395 Weilrod-Winden

centrifuge: type "Eppendorf", no. 5412,
test tubes: type "Eppendorf", polypropylene, no. 3810, Netheler und Hinz GmbH, D-2000 Hamburg 65

magnetic stirrer,
Cenco Deutschland GmbH,
D-5667 Haan

freezer: no. 8218,
Forma Scientific,
via Labotect,
D-3406 Bovenden

lyophiliser:
Speedvac concentrator
with cool trap, no. SVC 10011,
vacuum pump, no. RD4,
via Bachhofer,
D-7410 Reutlingen

Chemicals:

sodium acetate, no. 6257,
acetic acid, no. 63,
EDTA, no. 8418,
sodium chloride, no. 6404,

ethanol, no. 983,
magnesium chloride, no. 5833,
sodium hydroxide, no. 6498,
piperidine, no. 9724,
hydrazinium hydroxide, no. 804608,
E. Merck,
D-6100 Darmstadt 1

dimethylsulfate (DMS), no. 803071,
beta-mercaptoethanol, no. 805740,
Dr. Th. Schuchardt GmbH,
D-6100 Darmstadt 1

tris(hydroxymethyl)aminomethane (Tris),
no. T 1503,
sodium cacodylate, no. C 0250,
Sigma Chemie GmbH,
D-8024 Deisenhofen

calf thymus DNA, no. 104167,
tRNA, no. 109517,
Boehringer Mannheim GmbH,
D-6800 Mannheim 31

DMS buffer:

sodium cacodylate, pH 8.0	50	mmol/l
magnesium chloride	10	mmol/l
EDTA	1	mmol/l

DMS-stop buffer:	
sodium acetate, pH 7.0	1.5 mol/l
beta-mercaptoethanol	1 mol/l
tRNA	100 mg/l
hydrazine-stop buffer:	
sodium acetate, pH 7.0	0.3 mol/l
EDTA	0.1 mol/l
tRNA	25 mg/l
A-reaction buffer:	
NAOH	1.2 mol/l
EDTA	1 mmol/l
carrier DNA solution:	
calf thymus DNA	1 g/l

Procedure

Reference: according to Maxam and Gilbert (1980)

Storage: until determination:
-20 degrees centigrade

Base specific reactions: final concentration of components in assay mixture:

G-specific reaction:

labeled DNA	5 ul (up to 1 g DNA/l)
carrier DNA solution	1 ul (5 mg/l)
DMS	50 mmol/l
DMS buffer	194 ul

total assay volume: 200 ul

incubation time: 25 s at 37 degrees centigrade

termination of reaction by addition of 50 ul DMS-stop buffer

A-specific reaction:

labeled DNA	6 ul (up to 1 g DNA/l)
carrier DNA solution	1 ul (10 mg/l)
A-reaction buffer	93 ul

total assay volume: 100 ul

incubation time: 5 min at 90 degrees centigrade

termination of reaction by addition of 150 ul acetic acid (1 mol/l)

C + T-specific reaction:	labeled DNA	9 ul (up to 1 g DNA/l)
	carrier DNA solution	1 ul (17 mg/l)
	hydrazinium hydroxide	30 ul (10 mol/l)
	H2O	20 ul
	total assay volume: 60 ul	
	incubation time: 7 min at 37 degrees centigrade	
	termination of reaction by addition of 200 ul hydrazine-stop buffer	
C-specific reaction:	labeled DNA	5 ul (up to 1 g DNA/l)
	carrier DNA solution	1 ul (18 mg/l)
	sodium chloride	20 ul (1.4 mol/l)
	hydrazinium hydroxide	30 ul (10.7 mol/l)
	total assay volume: 56 ul	
	incubation time: 9 min at 37 degrees centigrade	
	termination of reaction by addition of 200 ul hydrazine-stop buffer	
Piperidine reaction (a):	piperidine	1 mol/l
	labeled and modified DNA	varying amounts (see individual reactions)
	total assay volume: 80 ul	
	incubation time: 25 min at 90 degrees centigrade	
	removal of piperidine by lyphilisation overnight, resuspension in 50 ul water and lyphilisation, repeat of lyphilisation step, resuspension in suitable volume water, storage at -20 degrees centigrade	

Scientific version: SOP BC 247/3
Text version: 2.Dec.86

(a) the same for all modification reactions

5.14 Polyacrylamide Gel Electrophoresis

Principle:	separation of DNA fragments according to their molecular size in an electrical field across a denaturing polyacrylamide gel
Time	
Sampling:	-
Determination:	after DNA labeling, within 1 half-life of the radioisotope used for labeling
Sample material and quantity:	DNA fragments, for example those generated by a sequencing reaction, 0.1 to 1 ug in total
Results expressed in:	-
Equipment:	electrophoresis apparatus: model SO, Bethesda Research Laboratories GmbH, D-6078 Neu-Isenburg power supply: model 2103, LKB Instrument GmbH, D-8032 Gräfelfing magnetic stirrer: IKA Combimag RCO, Janke und Kunkel GmbH und Co. KG, D-7813 Staufen test tubes: type "Eppendorf", polypropylene, no. 3810, Netheler und Hinz GmbH, D-2000 Hamburg 65 gel shaker: model 82421, Desaga GmbH, D-6900 Heidelberg 1 drying oven: Kottermann, no. 2716, via Faust GmbH, D-5000 Köln 90

Chemicals:

acetic acid, no. 63,
boric acid, no. 165,
EDTA, no. 8418,
glycerol, no. 4094,
bromophenol blue (BPB), no. 8122,
urea, no. 8487,
E. Merck,
D-6100 Darmstadt 1

trizma base (Tris), no. T 1503,
acrylamide, no. A 8887,
N,N'-methylene-bis-acrylamide,
no. M 7256,
N,N,N',N',-tetramethylethylene-
diamine (Temed), no. T 8133,
Sigma Chemie GmbH,
D-8024 Deisenhofen

sodium dodecylsulfate (SDS),
no. 20760,
ammonium persulfate (APS),
no. 13375,
Serva Feinbiochemika GmbH
und Co. KG,
D-6900 Heidelberg

bind-silane, no. 1850-251,
xylene cyanol (XC), no. 1840-502,
repel-silane (dimethyldichlorosilane),
no. 1850-252,
LKB Instrument GmbH,
D-8032 Gräfelfing

loading buffer:

formamide	800 ml/l
EDTA, pH 8.0	1 mmol/l
xylene cyanol	0.5 g/l
bromophenol blue	0.5 g/l

gel composition:

acrylamide	820 mmol/l
methylene-bis-acrylamide	13 mmol/l
boric acid	9.0 mmol/l
EDTA	2.5 mmol/l
Tris	90 mmol/l
ammonium persulfate	3.4 mmol/l
urea	8 mol/l
Temed	100 ml/l

Procedure:

according to Maxam and Gilbert (1980)
and Maniatis et al. (1982)

preparation of a polyacrylamide gel (60 g/l)

electrophoresis conditions:
prerun: 1500 V (a) for 1 h
run: 2000 V until bromophenol blue reaches the end of the gel

gel fixation:
30 min in acetic acid (100 g/l)

gel drying:
2 h in oven at 60 degrees centigrade

autoradiography:
for 18 h at room temperature

Scientific version: SOP BC 236/3
Text version: 3.Dec.86

5.15 Determination of Radioactivity (Liquid Scintillation Method)

Principle: determination of the light emission in mixtures of the radioactive sample with scintillation cocktail

Time: after precipitation of DNA and protein with ethanol and resuspension in TE solution

Sample material and quantity: resuspended DNA and protein pellets, 10 to 50 ul

Equipment: liquid scintillation counter:
Searle, Mark 3, model 6800,
W. Zinsser,
D-6000 Frankfurt/Main 50

counting vials: polyethylene,
with screw caps, no. 3000881,
Zinsser Analytik GmbH,
D-6000 Frankfurt/Main 50

Chemicals: scintillation cocktail: Instant Gel,
no. 6013009,
Packard Instrument Company,
D-6000 Frankfurt/Main

(a) corresponding to an electric field of approx. 43 V/cm

Procedure:	sample mixed with 4 ml scintillation cocktail and mixture equilibrated in the dark before counting automatic quench correction with experimentally determined correction standards counting time: variable, program: 2 (3H) and 3 (14C), cumulative counting (100000 cpm, 2 times, standard deviation: 2 0/0)
Scientific version:	SOP BC 148/2
Text version:	10.Feb.87

6 RESULTS AND DISCUSSION

6.1 Text

6.1.1 Preparation of microsomes

Cytochrome P-450 was induced by the intraperitoneal (i. p.) injection of Aroclor 1254 into rats at 500 milligrams/kilogram body weight. After 5 days, following starvation of the rats for 12 hours, the microsomes were prepared. Various methods were tried out before the method described in 5 METHOD was adopted. The methods used to prepare microsomes included simple pelletting at 9.8E2 m/s2 (100 x g) and resuspension in potassium chloride (SOP BC 104), purification in sucrose on discontinuous metrizamide gradients, the separation of smooth from rough microsomes in sucrose-cesium chloride gradients (SOP BC 267) and washing the microsomal pellet with potassium pyrophosphate. The best method was found to be pelletting of the microsomes in high salt (potassium hydrogen carbonate), resuspension in water to induce an osmotic shock, re-pelleting and resuspension of the microsomes in Tris-HCl, pH 7.5. This method, described in 5 METHOD, yielded microsomes with a sufficiently high AHM activity and a relatively low RNA and DNA nuclease content and was adopted as the standard method for this study. The AHM activities and RNA content of the microsomal preparations obtained by some of these methods are shown in TABLE 4. The DNA nuclease content of these microsomal preparations was estimated by agarose gel electrophoresis of DNA incubated with the various preparations, shown in FIGURE 2. All assays described in this documentation were carried out using microsomes prepared according to this method.

The effect of aurintricarboxylic acid (ATA), a nonspecific enzyme inhibitor (Bina-Stein and Tritton, 1976), which was formerly thought to be a specific inhibitor of DNA binding proteins (Blumenthal and Landers, 1973), and of the chelating agent EDTA, on DNA nuclease and microsomal activity was determined. In addition, the effect of RNase A-pretreatment of microsomes (in order to remove RNA) on their AHM activity was determined. The results indicate that preincubation of microsomes at 37 degrees centigrade, whether without or with RNase A, reduced their subsequent AHM activity by at least 30 percent (see TABLE 3). In addition, even low concentrations of ATA (10 micromoles/liter) significantly reduced AHM activity. The ATA concentration of 1 millimol/liter empirically determined to be required to eliminate DNA nuclease activity (data not shown) reduced the AHM activity of the microsomes by 94 percent. In view of these results RNase pretreatment and the use of ATA as a nuclease inhibitor were abandoned.

In contrast, the microsomes were found to tolerate relatively high levels of EDTA before AHM activity was significantly impaired (see TABLE 3). At 20 millimoles EDTA/liter the activity was reduced by 16 percent. At 5 millimoles EDTA/liter, the concentration found to be required to suppress DNA nuclease activity, the loss of microsomal AHM activity was only 4 percent. Henceforth all DNA incubations were performed in the presence of 5 millimoles EDTA/liter. The effectiveness of EDTA in suppressing DNA nuclease activity by chelating the divalent cations required by most DNA nucleases is shown in FIGURE 1. DNA was incubated with microsomes and either 5 millimoles EDTA/liter or 10 millimoles $MgCl_2$/liter for 1, 5, 20 and 60 minutes. After 5 minutes DNA nicking was observed in the samples containing $MgCl_2$. After 1 hour very little supercoiled DNA remained. In contrast, no significant nicking was observed in the samples containing EDTA.

6.1.2 Assay strategy

Initial experiments were carried out to determine the standard parameters for the DNA assays. The highest concentration of microsomal protein in the conformation assay which produced an acceptably low level of DNA nicking in the presence of 5 millimoles/liter EDTA was 0.1 grams/liter. This was subsequently used in all experiments. In the sequencing assay, DNA degradation was negligible at much higher concentrations of microsomal protein (see FIGURE 3) and experiments were performed at protein concentrations up to 5 grams/liter. NADPH was added at a concentration of 0.5 millimoles/liter except where the protein concentration was 1 gram/liter or higher, in which case the NADPH concentration was 2 millimoles/liter.

Each substance was assayed at 37 degrees centigrade at 3 concentrations at least, 2 or more incubation times and without or with heat posttreatment in the case of the conformation assay. The piperidine heat reaction was always carried out in the case of the sequencing assay.

The following substances were incubated in the presence of microsomal protein: DMNA and the PAH BA, DMBA, B(a)P and pyrene. Acridine, actinomycin D, BPDE, and formaldehyde were assayed directly. Catechol and CPP were assayed in either way.

For both assays, control samples containing all assay components without the test substance were always run to check for nuclease activity. The effect of the test substance was assessed relative to the control samples. The minimum effective concentration (MEC) was defined as the minimum concentration for a given substance at which DNA nicking was observed.

6.1.3 Activity of test substances in conformation and sequencing assay

6.1.3.1 B(a)P and BPDE

6.1.3.1.1 Conformation assay

BPDE was assayed at concentrations of 0.0001, 0.001, 0.005 and 0.01 millimoles/liter. Incubation times were 30 minutes.

The MEC for BPDE was 0.005 millimoles/liter. Higher concentrations caused increasing degradation of the DNA (see FIGURE 4). Heat posttreatment had no effect. The reaction of BPDE with DNA was very fast and was terminated after 15 minutes (data not shown).

B(a)P was assayed with microsomal activation at concentrations of 0.0001, 0.001, 0.008, 0.01, 0.08 and 0.1 millimoles/liter. Incubation times were 15 and 30 minutes. The results are shown in FIGURE 5.

The MEC for B(a)P, the parent compound of BPDE, was 0.001 millimoles/liter (see FIGURE 5B). More nicking was seen at 0.01 millimoles/liter but increasing the concentration to 0.1 millimoles/liter had no further effect. Heat posttreatment did not increase nicking. Most experiments were performed with B(a)P concentrations of 0.008 and 0.08 millimoles/liter. The effect at 0.08 millimoles/liter was stronger than that at 0.008 millimoles/liter (see FIGURE 5A), setting the concentration range for concentration-related effect at 0.001 to 0.080 millimoles/liter. The production of nicks was dependent on activation: no nicking was observed in the absence of microsomal protein (see FIGURE 5A). The reaction was terminated after 30 minutes.

6.1.3.1.2 Sequencing assay

BPDE was assayed at concentrations of 0.001, 0.0025, 0.005, 0.02 and 0.1 millimoles/liter. Incubation times were 2 and 15 minutes and 1 hour.

When BPDE-treated DNA was examined on a sequencing gel, a number of discrete bands were observed superimposed on low-level background radioactivity (see FIGURE 6). This showed that BPDE binds in a distinctly nonrandom fashion to DNA. Similar nonrandom binding to DNA has also been observed for BPDE by Boles and Hogan (1984) and by Sage and Haseltine (1984).

The MEC for BPDE in the sequencing assay was 0.0025 millimoles/liter, i. e. lower than in the conformation assay (see FIGURE 6). The lowest concentration at which all the observed adducts were present was 0.005 millimoles/liter. In contrast, Sage and Haseltine (1984), using short fragments of 44 and 94 bp as targets, were able to detect DNA strand breaks at a BPDE concentration as low as 0.25 milligrams/liter, corresponding to approx. 0.0008 millimoles/liter. This illustrates the increased sensitivity of a sequencing assay based on small fragments offering a limited number of target bases over that of the sequencing assay using the much larger target DNA (2700 bp) used in this study. At concentrations of up to 0.1 millimoles BPDE/liter, the DNA was progressively degraded and the bands became increasingly diffuse (see FIGURE 7). The latter was probably due to the presence in the DNA fragments of unremoved BPDE adducts which altered the mobility of the fragments.

The reaction of BPDE with DNA occurred very fast. Adducts were already visible after 2 minutes. Longer incubation periods resulted in more DNA breakdown but decreased intensity of the specific bands observed (see FIGURE 8).

Part of the sequence of pUR222 and the positions of the 7 identifiable adducts are shown in TABLE 5. All the adducts occurred within runs of 2 or more purines. 6 were at guanines and 1 at an adenine base.

It should be borne in mind that only alkali-labile adducts are detected by this assay. Sage and Haseltine (1984) estimated the number of alkali-labile BPDE adducts detected by sequencing analysis as 40 percent of the total number of adducts detected through HPLC analysis. Of these alkali-labile adducts 65 percent were at guanines, 23 percent at adenines and 12 percent at cytosines. Taking into consideration the fact that the number of adducts detected was too small to allow a detailed analysis, the results of this study are in agreement with the data of Sage and Haseltine (1984).

The major BPDE adduct has been generally reported to be an N2-guanine adduct (Jeffrey et al., 1979, 1980, Osborne et al., 1981). However, this adduct is not alkali-labile and thus the guanine adducts detected in sequencing analysis are probably N7 guanine adducts (Sage and Haseltine, 1984), which constitute approx. 20 percent of the total BPDE-DNA adducts (Osborne et al., 1981). A minor N3 adenine adduct has been found in vitro (Jeffrey et al., 1979, 1980). It has been postulated that lesions other than N2-substituted guanines could play a major role in the mutagenesis of B(a)P (Sage and Haseltine, 1984). The formation of apurinic or apyrimidinic sites at the sites of alkali-labile adducts usually leads to the insertion of adenine opposite the damaged site (e. g. Schaaper et al., 1982, Boiteux and Laval, 1982), causing base pair substitution mutations. The frequency of G → T, A → T and C → T substitutions (Eisenstadt et al., 1982) correlates well with the frequency of alkali-sensitive lesions at G, A and C (Sage and Haseltine, 1984).

B(a)P was assayed at a concentration of 80 micromoles/liter using both pUR222 and the PAL10 fragment as target DNA, with microsomal proteins at concentrations up to 5 grams/liter. No adduct formation was detected in the sequencing assay (data not shown).

6.1.3.1.3 Experiments with radioactively labeled B(a)P

14C- and 3H-labeled B(a)P were employed in the conformation assay to determine its binding to DNA and protein. In a preliminary experiment using (14C)B(a)P, 400 ul samples containing 3.7E4 bequerel (equivalent to 1 microcurie and 42.5 micromoles/liter) B(a)P and equal amounts (100 micrograms each) of DNA and protein were incubated for 1 hour at 37 degrees centigrade. The protein was extracted with phenol (see methods) and the protein and DNA were precipitated with ethanol from the phenol and aqueous phases respectively. The pellets were resuspended in buffer and counted. 10 times more radioactivity was found in the protein than in the DNA, indicating that the former is the preferred target for B(a)P binding. The amount bound to the DNA corresponded to 35 picomoles B(a)P. As 100 ug pBR322 DNA is about 35 picomoles, this implies that each DNA molecule contained on average 1 B(a)P adduct (data not shown).

To check the distribution of the radioactivity in the DNA, a sample was run on an agarose gel. The gel was subsequently dried and autoradiographed. Although at least 70 percent of the DNA was in the nicked form (a), as seen on the gel, the autoradiogram showed at least twice as much radioactivity in the supercoiled as in the nicked form (data not shown). This demonstrated that many B(a)P adducts do not cause DNA nicking and so will not be detected in the conformation assay.

(a) The observed nicking was probably due to DNA nuclease activity in the microsomal protein.

3H-labeled B(a)P, which has a higher specific activity than 14C-labeled B(a)P, was used in 2 subsequent experiments. In the 1st, the effect of protein concentration on the activation of B(a)P, and hence on its binding to DNA and protein, was investigated. 2 micrograms DNA were incubated with 3.7E5 bequerel (equivalent to 10 microcuries) (3H)-B(a)P, equivalent to a concentration of 40 micromoles/liter, and 0, 0.1, 0.5 or 2.5 grams/liter microsomal protein in a sample volume of 0.1 milliliters for 1 hour at 37 degrees centigrade. The binding of B(a)P to both protein and DNA was dependent on microsomal activation and increased with increasing protein concentration, reflecting the activation of increasing amounts of B(a)P (see TABLE 6). The rate of increase was, however, not linear. When the level of binding to protein or DNA was calculated per milligram substrate, a continuous decrease with increasing protein concentration was observed. This was expected due to the presence of more targets for B(a)P metabolites in the samples. Unexpectedly, the amount bound to DNA was consistently higher than that bound to protein when calculated as radioactivity per milligram substrate. This was in direct contrast to the experiment using 14C-labeled B(a)P, where 10 times more radioactivity was recovered in protein than in DNA. This discrepancy has not been explained. From the amount of radioactivity found in the DNA, the highest number of adducts was calculated to be approx. 9 per DNA molecule in the case of the sample containing 0.5 grams/liter protein. The number of adducts per DNA molecule for the sample containing 0.1 grams/liter protein was 3. As the conditions for this sample were identical to the standard conditions for the conformation assay, this implies that DNA treated with unlabeled B(a)P in this assay should also contain an average of 3 adducts. However, only a small proportion of the

DNA is nicked, as seen on agarose gels. It is therefore obvious that most B(a)P lesions do not cause DNA nicks under these conditions. This confirms the results of the experiment using 14C-labeled B(a)P and also the observations of Sage and Haseltine (1984).

In the 2nd experiment with 3H-labeled B(a)P, the effect of incubation time and of B(a)P and protein concentration on the binding of B(a)P to DNA and protein was investigated. Samples containing 2 micrograms DNA, 3.7E3, 3.7E4, 3.7E5 or 1.9E6 bequerel (3H)-B(a)P, equivalent to concentrations of 0.4, 4, 40 or 200 micromoles/liter B(a)P respectively, and 0, 0.1, 0.5 or 2.5 grams/liter microsomal protein were incubated for 2, 15 or 30 minutes and the amount of radioactivity bound to protein and DNA determined in each case. This experiment confirms that B(a)P must be activated by microsomal enzymes in order to bind to protein or DNA (see TABLE 7). Generally speaking, radioactivity bound to DNA increased with increasing incubation time and protein concentration. Appreciable radioactivity was bound after 2 minutes, showing the rapid activation of B(a)P, but binding continued to increase up to 1 h incubation time. As in the 1st experiment using (3H)-B(a)P, greater absolute amounts of radioactivity were recovered in protein than in DNA but the amount per milligram was consistently higher for DNA. Also as in the 1st experiment, binding to DNA was less at high (2.5 grams/liter) protein concentration, showing the competitive effect of increased protein concentration on B(a)P binding to DNA.

The amount of binding to DNA was maximal at 3.7E3 bequerel B(a)P per sample and a protein concentration of 0.1 grams/liter. Increasing either the B(a)P or protein concentration further did not substantially increase binding to DNA. In contrast, binding to protein increased with increasing protein and B(a)P concentration, although this increase was not linear.

The conclusion can be drawn that the benefits of increasing protein concentration in the assay - more activation of B(a)P - are offset by the increasing competition of protein with DNA for binding B(a)P. This confirms that the protein concentration used in the conformation assay - 0.1 grams/liter - is optimal not only in terms of reducing DNA nuclease activity but also in order to study DNA adduct formation.

6.1.3.2 DMBA

6.1.3.2.1 Conformation assay

DMBA was assayed with microsomal activation at 0.001, 0.01, 0.1 (data not shown) as well as 1 and 5 millimoles/liter. Incubation times were 15 minutes and 1 hour.

DNA nicking was observed at the 2 highest concentrations after 15 minutes incubation (see FIGURE 9). The effect was greater at 5 millimoles/liter than at 1 millimol/liter. Heat posttreatment and incubation for 1 h did not increase the effect. The MEC of DMBA was therefore 1 millimol/liter. In contrast, that of B(a)P, another carcinogenic PAH which, like DMBA, is activated by diol epoxide formation (reviewed by Singer and Grunberger, 1983), was 0.001 millimoles/liter, a factor of 1000 less, as described earlier. However, DMBA is a stronger carcinogen than B(a)P.

1 explanation for this difference between the results for B(a)P and DMBA could be that DMBA was insufficiently activated by the microsomal enzymes. It has been reported (Kornbrust and Dietz, 1985) that Aroclor 1254 poorly induces the metabolism of DMBA in rat liver. This may mean that only small quantities of active metabolites of DMBA were produced in these assays, hence the high MEC of DMBA. Sina et al. (1983) detected DNA strand breaks caused by both B(a)P and DMBA in hepatocytes from uninduced rats by the alkaline elution method. In their experiments, DMBA had a weaker effect than B(a)P at a lower concentration (0.03 millimoles/liter) and a stronger effect at a higher concentration (0.3 millimoles/liter). DNA repair systems in hepatocytes may also play a role in modifying the genotoxic effects of substances. Such experiments cannot therefore be directly compared with the in vitro tests reported here. In order to clarify these results, it would be necessary to assay DMBA using microsomes isolated from rats induced with some other substances (e. g. B(a)P or DMBA itself).

6.1.3.2.2 Sequencing assay

DMBA was tested at concentrations of 0.1, 1 and 5 millimoles/liter using both pUR222 and the PAL10 fragment as target DNA with microsomal protein at concentrations of 1 and 5 grams/liter. Incubation was for 15 minutes.

The results were negative: no adducts were seen (data not shown).

6.1.3.3 Pyrene

6.1.3.3.1 Conformation assay

Pyrene was assayed with microsomal activation at 0.001, 0.01, 0.1, 1 and 5 millimoles/liter. Incubation times were 15 minutes and 1 hour.

DNA strand breaks were seen at the lowest concentration of pyrene after 1 hour incubation (see FIGURE 10), giving an MEC for pyrene of 1 micromol/liter like B(a)P. After 15 minutes incubation, nicks were seen only from 0.1 millimoles/liter pyrene (see FIGURE 10). A concentration-dependent increase in nicking was not observed - the degree of nicking at 0.1 millimoles/liter was maximal. However, although it did not lower the MEC, heat treatment increased the amount of nicking seen at 0.1 millimoles/liter (data not shown).

This result for pyrene was unexpected. Pyrene is non-mutagenic in Salmonella typhimurium (McCann et al., 1975) and in mammalian cells (Huberman and Sachs, 1976, Bradley et al., 1981) and did not cause DNA strand breaks in hepatocytes in vitro (Sina et al., 1983). However, it is a potent cocarcinogen when applied together with B(a)P on mouse skin (Rice et al., 1984), and with B(a)P, beta-propiolactone and 60Co-radiation in a transformation assay (Baturay and Kennedy, 1986). The mechanism of the cocarcinogenic action of pyrene is not yet known.

6.1.3.3.2 Sequencing assay

Pyrene was tested at 0.1 and 1 millimoles/liter using both pUR222 and the PAL10 fragment as target DNA with microsomal protein at 0.1 and 0.5 grams/liter. Incubation time was 1 hour. No DNA adduct formation was observed (see FIGURE 7 for results with pUR222).

6.1.3.4 BA

6.1.3.4.1 Conformation assay

BA was tested with microsomal activation at 0.01 and 0.05 millimoles/liter. Incubation times were 15 and 30 minutes.

The results were negative - no DNA strand breaks were observed either without or with heat posttreatment (data not shown). Only these rather low concentrations were tested as BA has very low solubility in most solvents. However, a negative result was expected, as BA is noncarcinogenic and nonmutagenic, although it interacts with B(a)P by enhancing its mutagenicity at low doses and inhibiting it at higher doses (Hermann, 1981).

6.1.3.4.2 Sequencing assay

BA was tested at 0.01 and 0.05 millimoles/liter with microsomal protein at 1 and 5 grams/liter. Incubation time was 1 hour. No DNA adduct formation was observed (data not shown).

6.1.3.5 Catechol

6.1.3.5.1 Conformation assay

Catechol was assayed without microsomal activation at concentrations of 0.1, 1, 10 and 100 millimoles/liter, with activation at 1, 10 and 100 millimoles/liter. Incubation times were 15 minutes and 1 hour, up to 2 hours in some experiments, without microsomes, and 30 minutes with microsomes. As the main reported genotoxic activity of catechol is cocarcinogenicity (Van Duuren and Goldschmidt, 1976, Hecht et al., 1981) and comutagenicity (Yoshida and Fukuhara, 1983) with B(a)P, catechol was also assayed at concentrations of 0.1, 1, 10 and 100 millimoles/liter together with B(a)P at a concentration of 0.01 and 0.08 millimoles/liter with microsomal activation.

Assayed alone, catechol caused a concentration-related DNA nicking without and with microsomal activation (see TABLE 8 and FIGURES 5 and 11). The MEC of catechol was 0.1 millimoles/liter without

activation after 1 hour of incubation. No effect was seen after 15 minutes' incubation. The MEC with activation was 1 millimol/liter after 30 minutes' incubation. The reaction was slow and continued for at least 1 hour (data not shown). By 10 millimoles/liter the effect was maximal - no further increase was seen. Heat posttreatment increased the effect of catechol by a factor of 10 in the absence of microsomes (data not shown). In the presence of microsomes, catechol concentrations of 10 millimoles/liter and above were negative, although up to 1 millimol/liter was positive (see FIGURE 5). This was probably due to inhibition of microsomal enzymes by high catechol concentrations.

Catechol showed no increase in DNA nicking when assayed together with B(a)P (see FIGURE 5). At 100 millimoles/liter catechol even inhibited B(a)P-induced nicking. This was probably due to inhibition by this high catechol concentration of the microsomal enzymes required to activate B(a)P.

6.1.3.5.2 Sequencing assay

Catechol was tested without and with microsomal activation at concentrations of 0.001 to 10 millimoles/liter. Incubation time was 1 hour.

No specific adducts were seen with catechol and catechol had no effect in 4 experiments, including 1 experiment performed using the PAL10 fragment as target DNA (see TABLE 8). However, unspecific DNA breakdown was observed in 3 more experiments at various catechol concentrations. 1 of these experiments is shown in FIGURE 6. In this case DNA breakdown was observed at catechol concentrations of 0.1 and 10 millimoles/liter. A striking feature was that the concentrations which caused DNA breakdown varied between experiments. Moreover, as can be seen in FIGURE 6, the effect was not concentration-dependent. The reasons for this are not known.

As B(a)P was negative in the sequencing assay, catechol and B(a)P were not tested together. Instead, catechol and BPDE, the active metabolite of B(a)P, were tested together in the presence of microsomal protein at a concentration of 1 (data not shown) and 5 grams/liter. The results were negative - no adducts and no DNA breakdown were seen (see FIGURE 8). However, the incubation time of 1 hour also led to a general breakdown of DNA and the production of only weak adducts with BPDE alone. Moreover, BPDE binds to protein as well as to DNA. This probably explains why no DNA adduct formation due to BPDE was seen in the presence of microsomal protein.

6.1.3.5.3 Conclusion on results with catechol

The finding that catechol causes DNA strand breaks confirms the results of Yoshida and Fukuhara (1983), who found that catechol causes DNA strand breaks in vitro and in vivo. In the conformation assay, the MEC for catechol was 0.1 millimoles/liter. Heat post-treatment lowered the MEC by a factor of 10. The reaction was slow, continuing even after 1 hour incubation. Catechol did not enhance the nicking activity of B(a)P.

The results of the sequencing assay were difficult to interpret due to their irreproducibility. 4 experiments with catechol were negative, including that done using the PAL10 fragment as target DNA. In the remaining 3 experiments carried out, the catechol concentrations causing unspecific DNA breakdown varied between different experiments and the effect was not concentration-dependent. Further work to clarify the mechanisms of action of catechol is necessary to explain these results.

6.1.3.6 CPP

6.1.3.6.1 Conformation assay

CPP was assayed without and with microsomal activation at concentrations of 0.1, 1 and 10 millimoles/liter. Incubation was for 15 minutes and 1 hour. The results are shown in FIGURE 12.

Nicking was observed without and with activation at 10 millimoles/liter only, the highest CPP concentration. The effect was visible after 15 minutes incubation but much stronger after 1 hour incubation. Heat posttreatment reduced the MEC to 1 millimol/liter after 1 hour incubation without activation. The effect was much stronger in the absence of microsomal activation, with considerable DNA breakdown after longer incubation.

The finding that CPP nicked DNA in the absence as well as in the presence of microsomes was unexpected. As discussed in 4 INTRODUCTION, CPP must be activated by cytochrome P-450 to be mutagenic and carcinogenic (e. g. Hales, 1981). This further indicates that, as in the case of the PAH, DNA strand break formation may not always correlate with mutagenicity/carcinogenicity.

6.1.3.6.2 Sequencing assay

CPP was tested at concentrations of 1 and 10 millimoles/liter without and with microsomal activation. Microsomal protein was at concentrations of 0.1 to 5 milligrams/milliliter and the incubation time was 1 hour.

3 experiments were performed using pUR222 as the target DNA. Although 1 experiment gave negative results, 2 others showed specific adducts at high CPP concentration (see FIGURES 7 and 13).

In 1 experiment, the appearance of adducts was dependent on microsomal activation, with the highest concentration of microsomal protein (in this case, 2.5 grams/liter) yielding the strongest adducts (see FIGURE 7). In the 2nd experiment, however, CPP also yielded adducts in the absence of microsomal protein (see FIGURE 14). This agreed with the results of the conformation assay, where CPP caused DNA nicking without microsomal activation.

The adducts formed were all at guanines (see TABLE 5). This was as expected, as the predominant CPP adduct is at the N-7 of guanine (Kircher et al., 1979). However, in no case were the same positions attacked as were attacked by BPDE (see TABLE 5). As it has been argued earlier that the BPDE guanine adducts seen in the sequencing assay were also probably N-7 adducts, this difference in specificity between BPDE and CPP cannot be due to a difference in the positions at which adducts are formed but rather perhaps to effects of the flanking sequences. As in the case of BPDE, all CPP adducts occurred within purine runs.

A further sequencing assay was performed with CPP using the PAL10 fragment as target DNA. The results are shown in FIGURE 14. As in previous experiments, specific adducts were seen both without and with microsomal activation. All adducts observed in the absence of microsomes were also observed in the presence of microsomes. In the latter case many additional adducts were seen. Adduct formation was greater with microsomes at 0.5 grams/liter rather than with microsomes at 2.5 grams/liter, suggesting that protein may compete with DNA for the binding of CPP metabolites. In contrast to the results using pUR222 as target DNA, with PAL10 adducts were formed at all types of bases (see TABLE 9). No sequence specificity of adduct formation was apparent. This result is unexpected due to the fact that, as already mentioned, CPP mainly forms adducts at G (Kircher et al., 1979). In general far more

extensive DNA breakdown took place with CPP in this experiment than in those using pUR222 as target DNA. This confirms the expected increased sensitivity of the sequencing assay using a small DNA fragment as target compared with a larger plasmid DNA.

6.1.3.6.3 Experiments with radioactively labeled CPP

3 experiments using 3H-labeled CPP were performed in order to study the binding of CPP to DNA and protein. In 2 of them, no significant binding to either protein or DNA was observed (data not shown), possibly due to experimental errors. In the 3rd experiment, binding was observed. Samples contained 3.7E4 or 3.7E5 bequerel (equivalent to 1 or 10 microcuries) of 3H-labeled CPP (equivalent to a concentration of 0.005 or 0.05 millimoles/liter respectively), 2 micrograms DNA and 0, 0.1, 0.5 or 2.5 grams/liter microsomal protein. Incubation times were 15 minutes and 1 hour. The results are shown in TABLE 10. The level of binding to protein was generally low, i. e. little above background levels (taken as the radioactivity recovered from the phenol extracts in the absence of protein). Significant amounts bound to DNA. Expressed as radioactivity bound per milligram substrate, binding to DNA was greater than that to protein by approx. 10- to 1000-fold. Moreover, binding per milligram protein decreased with increasing protein concentration, whereas a concentration-dependent DNA binding was not observed. In contrast, B(a)P binding to both DNA and protein increased with increasing protein concentration and protein competed with DNA for protein binding, as discussed earlier. This implies that protein is not a good substrate for CPP, which contradicts the results for the sequencing assay using the PAL10 fragment as target DNA (see previous page).

CPP apparently also bound to DNA in the absence of microsomal activation, as also found in the conformation and sequencing assays. In general, more radioactivity bound at the higher CPP concentration than at the lower and more after 1 hour than after 15 minutes. The highest level of binding to DNA was at the higher CPP concentration with 0.5 grams/liter protein after 1 hour of incubation, although this value should be interpreted with caution as it is much higher than all other values.

The amount of radioactivity bound to DNA corresponded, in the case of the highest value, to 12 CPP adducts per DNA molecule. Under the standard conditions of the conformation assay (0.1 grams/liter protein and 1 hour incubation), the amount bound corresponded to 1 adduct per DNA molecule. The CPP concentration in this sample was 50 micromoles/liter, which was negative in the conformation assay. This suggests that, as in the case of B(a)P, most CPP adducts do not cause DNA strand breaks and cannot therefore be detected in the assays reported here.

6.1.3.6.4 Conclusion

CPP was positive in both the conformation and the sequencing assays. However, a high concentration (10 millimoles/liter) was required for a positive result and nicking and also adducts (in the sequencing assay) were seen both without and with microsomal activation. In the sequencing assay using the PAL10 fragment as target DNA, adducts were formed at all 4 bases. In experiments with 3H-labeled CPP, binding to protein was very low. Binding to DNA increased with increasing protein concentration but was also significant in the absence of protein. These results were unexpected as CPP requires metabolic activation for carcinogenic and mutagenic activity (e. g. Hales, 1981, Struck et al., 1971) and their significance is not clear.

6.1.3.7 DMNA

6.1.3.7.1 Conformation assay

DMNA was assayed with microsomal activation at concentrations of 0.01, 0.1, 1 and 10 millimoles/liter. Incubation was for 15 minutes. The results are shown in FIGURE 15.

A dose-dependent nicking of DNA was observed with a MEC of 0.1 millimoles/liter. The effect was maximal at 1 millimol/liter and did not increase further. DMNA required microsomal activation: when nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), required by the microsomal enzymes, was omitted, no nicking was observed. Heat posttreatment has no effect and incubating for 1 hour did not increase the effect (data not shown).

6.1.3.7.2 Sequencing assay

DMNA was assayed at concentrations of 0.1, 1, 5 and 10 millimoles/liter using both pUR222 and the PAL10 fragment as target DNA, with microsomal protein at concentrations of 0.1 and 0.5 grams/liter. Incubation was for 1 hour.

The results were negative. No DNA breakdown or adducts were observed (data not shown).

6.1.3.7.3 Experiments with radioactively labeled DMNA

Experiments were carried out using (14C)-labeled DMNA to attempt to determine the binding of DMNA to DNA and protein. In a pilot experiment 3.7E4 bequerel (equivalent to 1 microcurie) (14C)-DMNA (equivalent to a concentration of 0.7 millimoles/liter) was incubated with 1 microgram DNA without and with microsomal activa-

tion (protein at 0.1 grams/liter) in a final volume of 0.1 milliliters for 1 hour. Low amounts of radioactivity were recovered from both DNA and "protein" (the phenol phase) in both samples (data not shown). There was no difference between the samples without and with protein, suggesting that the observed binding was unspecific.

A 2nd, detailed experiment was then performed. 7.4E3 or 7.4E4 bequerel (equivalent to 0.2 or 2 microcuries and to 0.14 and 1.4 millimoles/liter respectively) (14C)-DMNA were incubated with 2 micrograms DNA in a final volume of 0.1 milliliters. Microsomal protein was present at concentrations of 0, 0.1, 0.5 or 2.5 grams/liter. Incubation times were 5, 20 and 60 minutes. The results are shown in TABLE 11.

Only in the samples with the protein concentration of 0.5 and 2.5 grams/liter was binding to protein above the background levels observed. Binding to DNA increased with incubation time but was in all cases approx. equal to the amount bound in the absence of microsomal protein. A certain amount of radioactivity was also recovered in samples containing no protein in the case of 3H-B(a)P and 3H-CPP, the other radioactive substances assayed (see TABLES 6, 7 and 10). In these cases, however, the binding in the presence of protein was significantly greater and a concentration-dependent and protein-dependent binding could be demonstrated. This was not the case for DMNA. It should be pointed out that the specific activity of the (14C)-DMNA was low. A binding frequency corresponding to 1 adduct per DNA molecule would yield only 20 decompositions per minute in the DNA pellet. Such a binding level would not be detected over the background level in this experiment. However, a binding rate to protein similar to that found for B(a)P should have been detectable in this experiment but was not observed. In short, these preliminary experiments yielded few useful data on the binding of DMNA to DNA and protein. No binding

was observed except for apparently unspecific binding both in the presence and in the absence of protein.

6.1.3.7.4 Conclusion

In general, the observed effects of DMNA in these assays were rather small. Although a positive result was obtained in the conformation assay (see FIGURE 15), the results of the sequencing assay were negative and the binding of (14C)-DMNA to protein and DNA was low (data not shown). A possible explanation for these results is that DMNA may have been insufficiently activated by the Aroclor-induced microsomes employed in this study. It has been reported by Kornbrust and Dietz (1985) that, as in the case of DMBA, DMNA metabolism in rat hepatocytes is not well induced by Aroclor. Further experiments using microsomes induced with other compounds (for example, B(a)P or phenobarbital) might yield better results for DMNA. However, the expected positive result was nevertheless obtained in the conformation assay, further indicating the ability of the test to predict genotoxicity.

6.1.3.8 Acridine

6.1.3.8.1 Conformation assay

Acridine was assayed at concentrations of 0.001, 0.01, 0.1 and 1 grams/liter without microsomal activation. Incubation was for 20 minutes and 1 hour.

The results were negative - no DNA nicking was observed, either without or with heat posttreatment (data not shown). This result was not unexpected in view of the fact that acridine is not mutagenic (Brown et al., 1980, Ferguson et al., 1985).

6.1.3.8.2 Sequencing assay

Acridine was assayed at concentrations of 0.001, 0.1 and 1 grams/liter in the absence of microsomes. Incubation was for 1 hour.

The results were negative - no DNA adducts or breakdown were observed (data not shown). This was in agreement with the results of the conformation assay.

6.1.3.9 Actinomycin D

6.1.3.9.1 Conformation assay

Actinomycin D was assayed at 0.001, 0.01, 0.1 and 0.2 grams/liter without microsomal activation (higher concentrations were not assayed due to the insolubility of the substance). Incubation was for 20 minutes and 1 hour.

The results were negative - no nicking was observed, either without or with heat posttreatment. A broadening of the DNA bands at actinomycin D concentrations of 0.01 grams/liter and above was seen, possibly indicating that the substance bound to the DNA without causing strand breaks (data not shown).

6.1.3.9.2 Sequencing assay

Actinomycin D was assayed at 0.001, 0.01, 0.05 and 0.2 grams/liter without microsomal activation. Incubation was for 1 hour.

The results were negative - no DNA adducts or breakdown were observed (data not shown). This agreed with the finding of the conformation assay that actinomycin D did not cause DNA strand breaks.

Sina et al. (1983) found that actinomycin D caused extensive DNA strand breaks, determined by the alkaline elution method, in rat hepatocytes at concentrations as low as 0.3 micromoles/liter, which is equivalent to about 0.0004 grams/liter. This is lower than the lowest concentration assayed in this study. This discrepancy could be due either to a greater sensitivity of the alkaline elution method relative to the assays employed here or to the fact that rat hepatocytes contain enzymatic systems which may have metabolised the substance. In this study, actinomycin D was assayed without microsomal activation. Further work is required to clarify this point.

6.1.3.10 Formaldehyde

6.1.3.10.1 Conformation assay

Formaldehyde was tested without and with microsomal activation at concentrations of 0.01, 0.1, 1, 10 and 100 millimoles/liter. Incubation was for 15 minutes and 1 hour.

Reproducible results could not be obtained for formaldehyde. 7 experiments without microsomal protein and 2 with were carried out. Of the former, 3 were positive, but the MEC varied between 0.01 and 0.1 millimoles/liter. The reaction was completed within 15 minutes. The remaining 4 experiments were negative. Both experiments with microsomal activation were negative. A further contradictory result was that of the positive experiments, heat posttreatment increased DNA nicking in 1 case but had no effect in the other 2 experiments (data not shown). The reason for this irreproducibility is not known.

6.1.3.10.2 Sequencing assay

Formaldehyde was tested at concentrations of 0.001, 0.01, 0.1, 1 and 10 millimoles/liter without microsomal activation. Incubation was for 1 hour.

As in the case of the conformation assay, the results of the sequencing assay for formaldehyde were inconclusive. 7 experiments were carried out. Of these, 2 were negative and the remaining 5 showed some DNA breakdown but no specific adducts (an example is shown in FIGURE 6). However, the formaldehyde concentration at which an effect was observed varied between 0.01 and 1 millimoles/liter (in the experiment shown in FIGURE 6, the positive concentrations were 0.1 and 1 millimoles/liter). Moreover, the effect was not concentration-dependent and higher concentrations than those producing an effect were negative (data not shown), suggesting that the positive results seen may be an artefact.

6.1.3.10.3 Conclusion

From the results of these assays it was not possible to register an unambiguous result for formaldehyde. Both assays yielded some positive and some negative results. The observation for the sequencing assay that, where 1 or 2 formaldehyde concentrations were positive, both higher and lower concentrations were negative, was similar to the corresponding assays for catechol, as described earlier.

As discussed in 4 INTRODUCTION, formaldehyde has been shown by the alkaline elution method to cause DNA strand breaks and DNA-protein crosslinks (Grafstrom et al., 1984). It might therefore have been expected to yield positive results in this study. The results obtained are difficult to explain and further work is required to clarify the situation.

6.1.4 General discussion

A number of substances were tested in order to validate the assays. The results of the conformation assay are summarized in TABLE 12. The main points to be considered are

(1) is it possible to assay substances requiring activation by microsomal enzymes?
(2) do the assays correctly detect genotoxicity of substances within the expected concentration range?
(3) are the results reproducible?
(4) how sensitive are the assays?

The methods used for preparing microsomes were successful in reducing the contamination with nucleases to an acceptable level. Residual nuclease activity remained. This was a problem only in the conformation assay and meant that microsomal protein could not be used at concentrations .GT.0.1 grams/liter. All assays were performed in the presence of 5 millimoles/liter EDTA to suppress nuclease activity. Under these conditions positive results were obtained for all genotoxic agents assayed which require metabolic activation. In the case of CPP, catechol and probably formaldehyde heat posttreatment lowered the MEC approx. 10-fold. Heat post-treatment had no effect on the MEC of the other substances assayed. This means that the conformation assay established for direct mutagens in a previous INBIFO study (P 0500/3060) could be extended to promutagens. The nuclease activity present in the microsomes did not disturb the sequencing assay, which could therefore be carried out with microsomes at much higher concentrations (up to 5 grams/liter were used). Despite this, CPP was the only substance which yielded DNA adducts with microsomal activation in the sequencing assay. The main reason for the insensitivity of the sequencing is probably the large size of the target DNA and the background DNA breakdown which makes it difficult to see low levels of adducts. Although more extensive DNA adduct formation and breakdown were observed for CPP in the

sequencing assay using the PAL10 fragment rather than the approx. 100 times larger pUR222 as target DNA, all other substances assayed (B(a)P, DMBA, pyrene, catechol and DMNA) gave negative results. The increase in sensitivity for the assay obtained from decreasing the size of the target DNA obviously did not suffice to produce positive results for most substances. It is therefore clear that, while the assay potentially yields useful and interesting data concerning the positions and nature of the attacked bases, most substances will not be able to produce any positive results using this assay. This fact greatly limits the usefulness of the sequencing assay for routine screening.

All the substances assayed, with the exception of acridine, are reported to have some form of genotoxic and/or carcinogenic activity. As discussed in 4 INTRODUCTION B(a)P, DMBA, CPP, DMNA and formaldehyde are carcinogenic and mutagenic. Catechol, pyrene and BA (at low concentrations) are comutagenic with B(a)P and catechol has been shown to cause DNA strand breaks. Actinomycin D is carcinogenic and causes DNA strand breaks.

All the assayed substances with the exception of BA, acridine and actinomycin D caused DNA strand breaks in the conformation assay. The negative results for these 3 substances were not surprising. BA and acridine are probably not genotoxic. Although DNA strand breaks were found by Sina et al. (1983) to be caused by actinomycin D, this is the only indication of genotoxic activity for this substance. The negative results for actinomycin D agree with the findings that it is nonmutagenic in bacterial and mammalian cells and is therefore probably not a genotoxic carcinogen.

4 PAH were tested. 2, B(a)P and DMBA, are carcinogenic and mutagenic. The other 2, pyrene and BA, are on the basis of the current knowledge noncarcinogenic and nonmutagenic, although pyrene is cocarcinogenic and BA comutagenic with B(a)P (see 4 INTRODUCTION). The results were in some respects unexpected. Concentration-related DNA nicking was observed for B(a)P and DMBA.

However, the MEC was greater for DMBA than for B(a)P by a factor of 1000, which does not correlate with the documented genotoxic potency of the 2 substances. As discussed earlier, this may be due to insufficient activation of DMBA by microsomal enzymes.

The binding studies carried out with B(a)P demonstrated a dose-related binding to DNA and protein. B(a)P adducts in supercoiled plasmid DNA could also be demonstrated, showing that by no means all adducts caused DNA strand breaks. Only adducts leading to DNA nicking can be detected in these assays. As expected, BA was negative for DNA strand breaks although it could be tested only at concentrations up to 0.05 millimoles/liter. However, pyrene was positive and had the low MEC of 1 micromol/liter, the same as for B(a)P. This again does not correlate with the known low genotoxicity of pyrene. Another problem with pyrene was that nicking activity was not concentration-related. Nicking was observed at the lowest assayed concentration (1 micromol/liter), however, did not increase upon increasing concentration (see FIGURE 10).

Taken together, the results of the assay are not a good indication of the genotoxic potential of PAH. Although DNA strand breaks were detected as expected for B(a)P, DMBA and pyrene, the degree of nicking and the observed MEC did not correlate with the potency of the substances described in other in vitro assays. As compared to published data from these other assays, the "activity" of DMBA was relatively weak, that of pyrene relatively strong. This suggests that the results of these assays must be interpreted with caution.

The concentrations of the various substances required to produce a positive effect posed more of a problem. As discussed in the results, the MEC for B(a)P and pyrene were less than that of DMBA by 3 orders of magnitude, although the latter substance is a more potent carcinogen than B(a)P and pyrene only has cocarcinogenic activity. A similar, though less pronounced, discrepancy

is shown by the results of the Salmonella/microsome assays for B(a)P and DMBA, where B(a)P is about 10 times as mutagenic as DMBA (McCann et al., 1975). Poor activation of DMBA by the Aroclor-induced microsomes used in this study may explain this discrepancy. Another possibility is that the in vitro activation of substances differs from the in vivo pathways. Different metabolites in vivo and in vitro were isolated in the case of DMBA (Bigger and Dipple, 1985) and of the PAH dibenzo(a,e)fluoranthene (Perin-Roussel et al., 1985). This underlines the fact that the results of all in vitro assays have to be interpreted with caution.

Catechol in itself was positive in the conformation assay. The MEC was 0.1 millimoles/liter. No positive effect of catechol together with B(a)P was seen in this study.

DMNA was positive in the conformation assay with an MEC of 0.1 millimoles/liter. This is comparable with the concentration of 0.3 millimoles/liter found by Sina et al. (1983) to cause DNA strand breaks in rat hepatocytes. The concentration required for a positive result in the Salmonella/microsome assays is much higher, at 130 to 270 micromoles/plate or around 50 to 150 millimoles/liter (McCann et al., 1975). Although DMNA gave a positive result, a number of experiments were negative and the binding experiment with 14C-labeled DMNA indicated that no DMNA binding to DNA or protein took place. Poor activation of DMNA by Aroclor-induced microsomes may explain the high concentrations of the substance required in these in vitro assays.

CPP was positive in the conformation test with an MEC of 10 millimoles/liter. This compares with the concentration range of 5 to

25 millimoles/liter used in the Salmonella/microsome assay (McCann et al., 1975). CPP was also the only substance (except BPDE) for which adducts were observed in the sequencing assay. A surprising result was that CPP was positive both without and with microsomal activation in both assays and also in binding experiments using 3H-labeled CPP. The reasons for this are not known.

Formaldehyde yielded the most unreproducible results. Although it was positive in the conformation assay with an MEC of 0.1 millimoles/liter in some experiments, in others it was negative or positive at different concentrations. In the sequencing assay no specific adducts were seen, but in some experiments unspecific DNA breakdown was observed at varying concentrations. Because of this irreproducibility formaldehyde cannot with certainty be scored as either positive or negative. This is the only case for which an equivocal result was obtained.

The assays were in general only conditionally reproducible. Many substances gave negative results in some experiments and positive in others. Most of the results could only be qualitatively interpreted and some substances, while yielding a positive result, did not show concentration-dependence of the amount of DNA strand breaks generated (see 5 RESULTS). This meant that in many cases different experiments with the same substance could not be compared with each other. This inconsistency appears to be inherent in the assay system and is probably due to the many different factors affecting the results such as the behavior of the microsomes and the DNA preparation. However, qualitatively positive results were obtained in the conformation assay for almost all substances at concentrations comparable to those required in other standard in vitro assays, showing that the test can usefully be applied to suspected genotoxic agents.

In terms of the MEC of the assayed substances in the assays described in this study, the sensitivity of the assays was comparable to or in many cases greater than that of the Salmonella/microsome mutagenicity assay. Greater sensitivity of the assays used with respect to the Salmonella assay would be expected from the fact that mutations result from the action of error-prone repair processes (reviewed by Witkin, 1976) and lesions repaired by error-free mechanisms have no genetic consequences. This point also at least partly explains the lack of correlation in many cases between DNA adduct formation by a substance and its carcinogenicity and must always be borne in mind when evaluating DNA adduct formation.

The sensitivity of the assays can also be considered in terms of the number of DNA adducts capable of being detected. This cannot be quantitated for the sequencing assay as background DNA breakdown affects the results. In the case of the conformation assay, the sensitivity depends in part on the size of the plasmid DNA employed. 1 DNA strand break per molecule will convert the molecule from the CCC to the OC form. A substance causing 1 strand break per 50000 bp will change the conformation (assuming equal distribution of strand breaks) of 100 percent of the molecules of a plasmid 50000 bp in size but only 10 percent of those of a plasmid 5000 bp in size. An additional factor to be taken into account is the minimum change in the relative proportions of the 2 plasmid forms which is detectable on an agarose gel. This is governed by the powers of resolution of the eye (for subjective evaluation) and of densitometry (for objective evaluation). The latter would be the more favourable method for quantification of the 2 DNA conformation forms because (1) slight differences of the relative proportions of the 2 forms cannot be detected by eye but by high resolution densitometry, and (2) the absolute amount of the DNA recovered on the gel, which is objectively

evaluated by densitometry, would give more information of the effects exerted to DNA by a certain substance, i. e. if there is crosslinking activity. As a rough estimate, it may be assumed that at least 5 percent of the molecules must be nicked in order to detect a change on an agarose gel by eye. This gives a sensitivity for pBR322, which is 4563 bp in size (Sutcliffe, 1979), of 1 in 4563 x 5 percent or approx. 1 adduct in E5 bp. Using a larger plasmid would increase the sensitivity of the assay in direct proportion to the increased size of the plasmid. However, this is impractical as large plasmids (e. g. .GT.15.000 bp) are difficult to prepare and handle.

The sensitivity of the sequencing assay was increased by reducing the size of the assayed DNA (from 2700 bp approx. for pUR222 to approx. 230 bp for the PAL10 fragment) to lower the number of target bases available for adduct formation. Similar sequencing assays reported by other workers have always involved small fragments of between 40 and 480 bp (Sage and Haseltine, 1984, Inouye, 1984, Bichara and Fuchs, 1985). However, as discussed earlier, most substances were negative also using this small fragment showing that the assay remained too insensitive in most cases.

Many different CCC DNA have been used to assay DNA strand breaks in assays similar to the conformation assay, including the mammalian virus SV40 (Fahl et al., 1984), the bacteriophage ØX174 (Wakata et al., 1985) and the plasmids pBR322 (Inouye, 1984, Sawadaishi et al., 1985) and PM2 (Borish et al., 1985). In some cases the number of strand breaks were quantitated by densitometric scanning of the negatives of gel photos (e. g. Borish et al., 1985). The sensitivity of these assays was comparable to that of the conformation assay.

The 3 other main DNA assays described in the literature are the assaying of DNA adducts by immunological or HPLC methods,

the assaying of DNA strand breaks by alkaline elution and the detection of DNA adducts by the 32P postlabeling method (reviewed by Sina et al., 1983, Lohman et al., 1985, Jeffrey, 1985). Immunological methods detect 1 adduct in E7 bases or more and have been used to detect B(a)P adducts in humans and animals (e. g. Perera et al., 1982, Harris et al., 1985) but only specific DNA lesions can be detected by antibodies directed against this lesion. Similarly only specific adducts for certain substances such as B(a)P can be detected by HPLC methods (e. g. Rahn et al., 1982, Harris et al., 1985). The alkaline elution method also detects 1 strand break in E7 bases (reviewed by Kohn et al., 1981). However, quantitation is difficult and only strand breaks, not adducts, are recorded. The highest sensitivity is achieved by the 32P postlabeling method, by which in general 1 adduct in E7 bases is detected (Jeffrey, 1985), although levels as low as 1 adduct in E10 bases have been detected (Lu et al., 1986, Reddy and Randerath, 1986). However, this method works best with hydrophobic substances but is much less sensitive when simple alkylating agents are used (Jeffrey, 1985). An elaborate separation technique is also required in order to separate adducts from the vast bulk of unmodified bases.

In conclusion, the 2 assays described in this study are simple to perform, capable of predicting genotoxic activity of a given substance and (in the case of the sequencing assay) yield useful information concerning the mechanism of adduct formation. The sensitivity of the assays is comparable to that of the widely-used Salmonella/microsome assay. However, the methods can only be used in vitro and other methods for the assaying of DNA adducts are available which, while each has its own particular disadvantage, are capable of assaying for adducts in vivo and at a sensitivity at least 2 orders of magnitude greater than that of these assays. Despite this, these assays are a useful tool in the assaying of DNA adduct formation and are applicable to complex mixtures such as cigarette condensate.

6.2 Tables and Figures

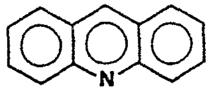
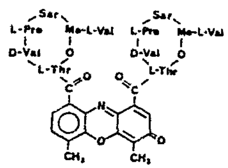
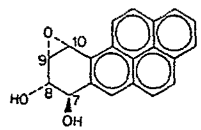
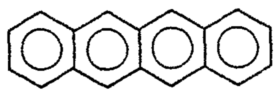
TEST SUBSTANCE	SYNONYM	STRUCTURE	CHEMICAL ABSTRACTS SERVICE REGISTRY NO.	SOURCE	DATE OF RECEIPT AT INBIFO	STORAGE
acridine	2,3,5,6 di-benzo-pyridine		290-94-6	Sigma, D-8024 Deisenhofen	18.Dec.85	approx. 4 degrees centigrade
actinomycin D	-		50-76-0	Sigma, D-8024 Deisenhofen	18.Dec.85	approx. 4 degrees centigrade
BPDE	7-beta,8-alpha-dihydroxy-(9-alpha, 10-alpha)-epoxy-7,8,9,10-tetrahydro-benzo(a)pyrene, anti-(+)-benzo(a) pyrene-7,8-diol-9,10-epoxide		58917-67-2	ITT Research Institute, Chicago, USA	approx. 1984	-30 degrees centigrade
2,3 benz-anthracene	naphthacene, benz(b)anthracene, tetracene		92-24-0	Sigma, D-8024 Deisenhofen	21.Feb.86	approx. 20 degrees centigrade

TABLE 1

GENERAL INFORMATION ON TEST SUBSTANCES EXAMINED

Personal Notes

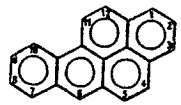
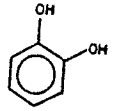
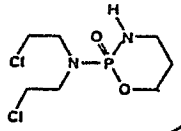
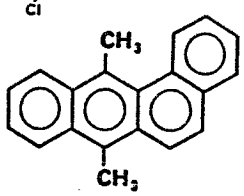
TEST SUBSTANCE	SYNONYM	STRUCTURE	CHEMICAL ABSTRACTS SERVICE REGISTRY NO.	SOURCE	DATE OF RECEIPT AT INBIFO	STORAGE
benzo(a) pyrene	3,4-benzpyrene		50-32-8	Serva, D-6900 Heidelberg	28.Aug.82	approx. -20 degrees centigrade, light protected
catechol	1,2-dihydroxy-benzene, brenz-katechin, pyrocatechol		120-80-9	Sigma, D-8024 Deisenhofen	27.Nov.85	approx. 20 degrees centigrade
cyclo-phosphamide	Endoxan		6055-19-2	Sigma, D-8024 Deisenhofen	27.Feb.86	approx. 4 degrees centigrade
7,12-dimethyl-benz(a)anthra-cene	9,10-dimethyl-1,2-benz(a)anthracene, 1,4-dimethyl-2,3-benephenun-threne		57-97-6	Sigma, D-8024 Deisenhofen	21.Feb.86	approx. 4 degrees centigrade

TABLE 1 (continued)

GENERAL INFORMATION ON TEST SUBSTANCES EXAMINED

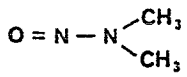
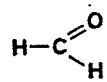
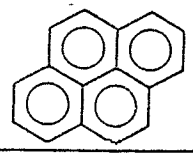
TEST SUBSTANCE	SYNONYM	STRUCTURE	CHEMICAL ABSTRACTS SERVICE REGISTRY NO.	SOURCE	DATE OF RECEIPT AT INBIFO	STORAGE
dimethyl-nitrosamine	N-nitrosodimethyl-amine, N-methyl-N-nitrosomethanamine		62-75-9	Sigma, D-8024 Deisenhofen	27.Feb.86	approx. 4 degrees centigrade,
formaldehyde solution	formalin, formol, morbicid		50-00-0	E. Merck D-6100 Darmstadt	23.Jan.86	approx. 21 degrees centigrade
pyrene	benzo(def)phen-anthracene		129-00-0	Sigma, D-8024 Deisenhofen	21.Feb.86	approx. 4 degrees centigrade

TABLE 1 (continued)

GENERAL INFORMATION ON TEST SUBSTANCES EXAMINED

Personal Notes

TEST SUBSTANCE	RELATIVE MOLECULAR MASS	APPEARANCE	DENSITY	SOLUBILITY
acridine	179.22	rhombic or orthorhombic	1.005	soluble in alcohol, ether
actinomycin D	1255.50	white crystals	"	soluble in acetone
BPDE	302.00	yellow needles	"	soluble in acetone
2,3 benz-anthracene	228.29	orange-yellow leaflets	1.35	soluble in acetone
benzo(a)pyrene	252.32	yellowish plates or needles	"	soluble in acetone, benzene, toluene, alcohol
catechol	110.11	monoclinic tablets	1.344	soluble in water, alcohol, chloroform
cyclophosphamide	279.10	white crystals	"	soluble in water
7,12-dimethyl-benz(a)anthracene	256.35	faint greenish-yellow plates, leaflets	"	soluble in acetone
dimethyl-nitrosamine	74.08	yellow liquid	1.005	very soluble in water, alcohol, ether
formaldehyde solution	30.03	colorless liquid	1.081	soluble in water, alcohol, acetone
pyrene	202.24	colorless mono-clinic prismatic tablets	1.271	soluble in acetone

TABLE 2

PHYSICAL AND CHEMICAL PROPERTIES OF TEST SUBSTANCES EXAMINED

SAMPLE	3- plus 9-HYDROXY-B(a)P (umol/(g x h))	(0/0)
control	95.64	100
+ 0.01 mmol ATA/l	70.40	74
+ 0.1 mmol ATA/l	27.20	28
+ 1 mmol ATA/l	6.16	6
+ 5 mmol EDTA/l	92.00	96
+ 20 mmol EDTA/l	80.56	84
preincubation with RNase A	62.00	65

TABLE 3

EFFECT OF ATA, EDTA AND PREINCUBATION AT 37 DEGREES CENTIGRADE FOR 30 MIN WITHOUT AND WITH RNASE A ON MICROSOMAL AHM ACTIVITY

Remarks: experimental conditions: induced rat liver microsomes in 100 mmol potassium phosphate/l pH 7.4, B(a)P concentration: 0.08 mmol/l, RNase A: 25 mg/l

PREPARATION	3- plus 9-HYDROXY-B(a)P (umol/(g x h))	(0/0)	RNA (mg/g protein)
pelletting 981 m/s2 (= 100 x g)	68.8	100	n. d.
metrizamide	114.0	166	n. d.
"smooth" microsomes from sucrose-cesium-chloride gradient	95.8	139	15.21
"rough" microsomes from sucrose-cesium-chloride gradient	78.0	113	18.29
pyrophosphate	99.7	145	2.67
bicarbonate	102.9	150	2.13

TABLE 4

AHM ACTIVITY AND RNA CONTENT OF MICROSOMES PREPARED BY DIFFERENT METHODS

Remarks: experimental conditions: as in TABLE 3
n. d.: not detected

Sequence of pUR222

```
T T G C A G C A C T

G A C C C T T T T G
  1 1
G G A A C G C A A T

G G G T T G A A T T

A G C G G A A C G T
                2 2 2
C G T G T A G G G G
2       2   2 2
G A A A G C G G T C
2
G A C C G C A T T A

T C G C T T C T C C
1 1 1       1 1
G G G C G T G G C T

A G C G G G A A G G
```

TABLE 5

SITES OF DNA ADDUCTS WITH BPDE AND CPP

Remarks: 1: BPDE
2: CPP

PROTEIN CONCENTRATION (g/l)	RADIOACTIVITY (dpm x E3) PROTEIN	DNA	SPECIFIC RADIOACTIVITY (dpm x E3/mg) PROTEIN	DNA
0	0.1 (a)	0.07	-	35
0.1	12.5	11.7	1250	5850
0.5	26.9	30.9	538	15450
2.5	61.6	15.9	246	7950

TABLE 6

(3H)-B(a)P BINDING TO PROTEIN AND DNA (Experiment 1)

Remarks: Samples contained 2 ug DNA in a final volume of 0.1 ml and were incubated for 1 h at 37 degrees centigrade.

(a) This amount of radioactivity was recovered from the phenol phase of the extracted sample which in this case contained no protein.

B(a)P	INCUBATION TIME	PROTEIN CONC.	RADIOACTIVITY (dpm x E3)		SPECIFIC RADIOACTIVITY (dpm x E3/mg)	
			DNA	PROTEIN	DNA	PROTEIN
(Bq)	(min)	(g/l)				
3.7E3	2	-	0.06	0.05 (a)	30	-
	15	-	0.08	0.08 (a)	40	-
	30	-	0.11	0.05 (a)	55	-
	2	0.1	0.67	0.18	335	18
	15	"	1.61	0.16 (b)	805	16 (b)
	30	"	2.32	0.97	1150	97
	2	0.5	0.38	0.85	190	17.0
	15	"	0.52	0.33	260	6.6
	30	"	0.90	0.57	450	11.4
	2	2.5	0.25	2.01	125	8.0
	15	"	0.17	2.19	85	8.8
	30	"	0.25	2.01	125	8.0
3.7E4	2	-	0.08	0.08	40	-
	15	-	0.05	0.05	25	-
	30	-	0.09	0.09	45	-
	2	0.1	0.57	0.20	285	20
	15	"	3.25	0.77	1625	77
	30	"	3.30	0.50	1650	50
	2	0.5	0.51	0.32	255	6.4
	15	"	2.15	0.97 (b)	1075	19.4 (b)
	30	"	3.65 (b)	0.67	1825 (b)	13.4
	2	2.5	0.51	8.34	255	33.4
	15	"	0.82	13.45	410	53.8
	30	"	2.68	16.36	1340	65.4

TABLE 7

RADIOACTIVITY BOUND TO PROTEIN AND DNA FOLLOWING INCUBATION WITH 3H-LABELED B(a)P

Remarks: Samples contained 2 ug pBR322 DNA in a final volume of 0.1 ml.

(a) These amounts of radioactivity were recovered from the phenol phases of the extracted samples, which in these cases contained no protein.
(b) single values

B(a)P (Bq)	INCUBATION TIME (min)	PROTEIN CONC. (g/l)	RADIOACTIVITY (dpm x E3) DNA	RADIOACTIVITY (dpm x E3) PROTEIN	SPECIFIC RADIOACTIVITY (dpm x E3/mg) DNA	SPECIFIC RADIOACTIVITY (dpm x E3/mg) PROTEIN
3.7E5	2	-	0.07	0.06	35	-
	15	-	0.17	0.08	85	-
	30	-	1.07	0.21	535	-
	2	0.1	0.30	0.10	150	10
	15	"	1.52	0.31	760	31
	30	"	4.42	0.75	2210	75
	2	0.5	0.82	0.26	410	5.2
	15	"	1.32	0.30	660	6.0
	30	"	4.22	1.87 (a)	2110	37.4 (a)
	2	2.5	1.60	4.89	800	19.6
	15	"	0.33	8.86	165	35.4
	30	"	1.93	17.93	965	71.7
1.9E6	2	-	0.12	0.07	60	-
	15	-	0.73	0.13	365	-
	30	-	3.03	0.66	1515	-
	2	0.1	0.59	0.15	295	15
	15	"	1.87	0.91	935	91
	30	"	3.09	0.60	1545	60
	2	0.5	1.14	0.18	570	3.6
	15	"	1.05	0.31	525	6.2
	30	"	3.09	0.93	1545	18.6
	2	2.5	1.10	9.78	550	39.12
	15	"	0.40	23.00	200	92.0
	30	"	1.28	22.22	640	88.9

TABLE 7 (continued)

RADIOACTIVITY BOUND TO PROTEIN AND DNA FOLLOWING INCUBATION WITH 3H-LABELED B(a)P

Remarks: Samples contained 2 ug pBR322 DNA in a final volume of 0.1 ml.

(a) single values

EXPERIMENT	CATECHOL CONCENTRATION (mmol/l)	TYPE OF ASSAY (a)	MICROSOMAL ACTIVATION	MEC (mmol/l) -HEAT	+HEAT	NEGATIVE CONC.
1	0.01 to 10	C	0	0.1	0.01	none
2	0.01 to 10	"	"	0.1	0.01	"
3	0.1 to 100	"	"	0.1	n.d.	"
4	1 to 100	"	+	1	1	"
			0	10	1	"
5	0.1 to 100	"	+	1	1	10, 100
6	0.001 to 10	S	0	-	0.1	0.001, 0.01, 10
7	0.001 to 10	"	"	"	1	0.001, 0.01, 0.1
8	0.001 to 10	"	"	"	0.1	0.001, 0.01, 1
9	0.1 to 10	"	+	"	0	all assayed
			0	"	"	"
10	0.1 to 0.5	"	+	"	"	"
			0	"	"	"
11	0.1 to 0.5	"	+	"	"	"
			0	"	"	"
12	0.1 to 10	" (b)	+	"	"	"
			0	"	"	"

TABLE 8

RESULTS OF DIFFERENT EXPERIMENTS WITH CATECHOL

Remarks: n. d.: not detected

(a) C = conformation, S = sequencing
(b) target DNA was the PAL10 fragment

Sequence of PAL10 Fragment

```
2         2 2 2 1 2 2 1 2 2 2           2 2
T T T C A A A C G A T G T C A A A A A C G G T T C

1   2               1 1     1   1             1 2
G A C T A C A A C G G T T G A G T A C T A C G T T

    2 2   2 2     1           2               1
C T C C A A T A A G A C T A T A C C C A C A C G T

1   1 2
G C G G A A A T A A C A T G G G A T T G A T T C
```

TABLE 9

SITES OF DNA ADDUCTS WITH CPP

Remarks: 1: cyclophosphamide adducts both without and with microsomal activation
2: cyclophosphamide adducts with microsomal activation

3H-CPP (umol/l)	PROTEIN CONC. (g/l)	INCUBATION TIME (min)	RADIOACTIVITY (dpm x E3) DNA	RADIOACTIVITY (dpm x E3) PROTEIN	SPECIFIC RADIOACTIVITY (dpm x E3/mg) DNA	SPECIFIC RADIOACTIVITY (dpm x E3/mg) PROTEIN
5	0	15	1.1	0.1 (a)	550	-
	"	60	1.8	0.2 (a)	900	-
50	"	15	1.5	1.4 (a)	750	-
	"	60	3.0	1.8 (a)	1500	-
5	0.1	15	0.6	0.3	300	30
	"	60	1.0	0.7	500	70
50	"	15	3.4	1.6	1700	160
	"	60	2.6	1.2	1300	120
5	0.5	15	1.2	0.3	600	6
	"	60	1.5	0.6	750	12
50	"	15	1.6	1.0	800	20
	"	60	33.7	2.9	16850	58
5	2.5	15	0.5	2.4	250	10
	"	60	3.4	1.4	1700	6
50	"	15	8.4	0.6	4200	2
	"	60	6.7	5.5	3350	22

TABLE 10

BINDING OF (3H)-CPP TO DNA AND PROTEIN

Remarks: Samples contained 2 ug DNA in a final volume of 0.1 ml.

(a) This amount of radioactivity was recovered from the phenol phase of the extracted samples, which in these cases contained no protein.

DMNA (mmol/l)	INCUBATION TIME (min)	PROTEIN CONC. (g/l)	RADIOACTIVITY (dpm x E3) DNA	PROTEIN
0.14	5	0	0.20	0.22 (a)
	20	"	0.24	0.17 (a)
	60	"	0.53	0.21 (a)
1.4	5	"	0.53	0.26 (a)
	20	"	0.69	0.26 (a)
	60	"	3.88	0.21 (a)
0.14	5	0.1	0.18	0.20
	20	"	0.22	0.19
	60	"	0.48	0.20
1.4	5	"	0.30	0.23
	20	"	0.30	0.23
	60	"	2.97	0.30
0.14	5	0.5	0.20	0.20
	20	"	0.18	0.25
	60	"	0.48	0.24
1.4	5	"	0.32	0.25
	20	"	0.40	0.29
	60	"	3.26	0.32
0.14	5	2.5	0.23	0.37
	20	"	0.26	0.41
	60	"	0.56	0.56
1.4	5	"	0.60	1.43
	20	"	0.98	2.21
	60	"	4.19	1.84

TABLE 11

BINDING OF 14C-LABELED DMNA TO DNA AND PROTEIN

(a) recovered from the phenol phases

SUBSTANCE	CONC. TESTED (mmol/l)	METABOLIC ACTIVATION	DNA STRAND BREAKS	MINIMUM EFFECTIVE CONCENTRATION (mmol/l)	EFFECT OF HEAT POST-TREATMENT
BPDE	0.001 to 0.1	0	+	0.005	0
B(a)P	0.0001 to 0.1	+	+	0.001	0
B(a)P + catechol	0.08 1 to 100	+	+	0.001 (a)	-
DMBA	0.001 to 5	+	+	1	0
DMNA	0.01 to 10	+	+	0.1	0
pyrene	0.001 to 5	+	+	0.001	(+) (b)
BA	0.01, 0.05	+	0	-	-
catechol	0.01 to 100	0, +	+, +	0.1 (c), 1 (d)	+
CPP	0.1 to 10	0, +	+, +	10, 10	+
formaldehyde	0.01 to 10	0	+?	0.1?	+? (e)
acridine	0.006 to 5.6	0	0	-	-
actinomycin D	0.0008 to 0.159	0	0	-	-

TABLE 12

RESULTS OF THE CONFORMATION ASSAY

(a) same value as for B(a)P alone
(b) increased effect but no lowering of MEC
(c) incubation time was 60 min
(d) incubation time was 30 min
(e) 1 experiment, 2 others showed no effect

SUBSTANCE	MUTAGENICITY IN SALMONELLA		EXPECTED		INBIFO RESULTS (P 0500/5075)	
	S9 ACTIVATION		DNA STRAND BREAK ACT.	DNA ADDUCTS	MICROSOMAL ACTIVATION	
	WITHOUT	WITH			WITHOUT	WITH
actinomycin D	0 (a)	0 (a)	+ (b)	+ (c,d,e)	0	-
acridine	- (f,g)	- (f,g)	- (h)	- (i)	0	-
BA	-	+ (g)	+ (j)	-	-	0
B(a)P	0 (g)	+ (g)	+ (k)	+ (l)	-	+
catechol	0 (m)	0 (m)	+ (n)	0 (o)	+	+
CPP	0 (g)	+ (g)	+ (p)	+ (q)	+	+
DMBA	0 (g)	+ (g)	+ (k)	+ (l)	-	+
DMNA	-	+ (g)	-	+ (c)	-	+
HCHO	+ (r)	+ (r)	+ (s)	+ (t)	+ (?)	-
pyrene	0 (g)	0 (g)	0 (b)	0 (u)	-	+

TABLE 13

COMPARISON OF PUBLISHED RESULTS OF IN VITRO GENOTOXICITY ASSAYS WITH RESULTS OF CONFORMATION ASSAY IN PRESENT STUDY

(a) Bruce and Heddle (1979)
(b) Sina et al. (1983)
(c) Hsu et al. (1986)
(d) 7-(2,3-epoxypropoxy)actinomycin D (= EPA), EPA: DNA adducts, Sengupta et al. (1984)
(e) N2-(3'-aminopropyl)-AMD (= NAPA), NAPA: DNA adducts, Sengupta, S.K.
(f) acridine orange, without S9 negative, with S9 positive
(g) Kier (1986)
(h) benz(a)acridine and benz(c)acridine induce no DNA strand breaks, Carcinogenesis 6 : 455-457 (1985), diacridines produce single strand breaks, Biochem. J. 226 : 175-182 (1985), acridine derivatives produce DNA strand breaks, Proc. Natl. Acad. Sci. 81 : 1361-1365 (1984)
(i) certain acridine dyes bound to DNA, Prusik, T. (1981)
(j) Cooper et al. (1983)
(k) Schechtman et al. (1982)
(l) DiGiovanni et al. (1986)
(m) Yoshida and Fukuhara (1983)
(n) Yamada et al. (1985)
(o) catechol: increase of B(a)P: DNA adducts, Melikian et al. (1986)
(p) Skare and Schrotel (1984)
(q) Kircher et al. (1979)
(r) TA100 positive, Environ. Mutagenesis Suppl. 1 : 96 (1983)
(s) Grafstrom et al. (1984)
(t) Heck and Casanova-Schmitz (1983)
(u) pyrene: increase of B(a)P: DNA adducts, Rice et al. (1984)

DOCUMENTATION P 0500/5075 URO12RB13 7042 M

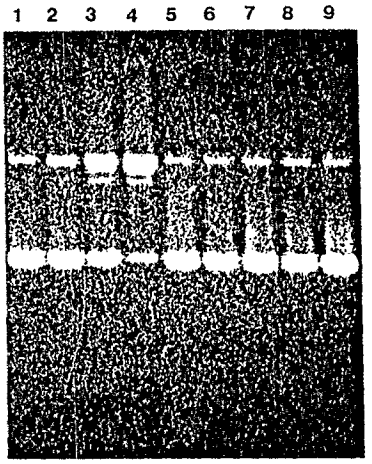


FIGURE 1

EFFECT OF MgCl2 AND EDTA ON NUCLEASE ACTIVITY

Remarks: Lanes 1 to 4: DNA incubated with microsomes and MgCl2 (10 mmol/l) for 1, 5 and 20 min and 1 h respectively

Lanes 5 to 8: DNA incubated with microsomes and EDTA (5 mmol/l) for 1, 5 and 20 min and 1 h respectively

Lane 9: control (untreated DNA)

Protein concentration was 0.1 g/l, DNA concentration 20 mg/l.

DOCUMENTATION P 0500/5075 URO12RB14 7042 M

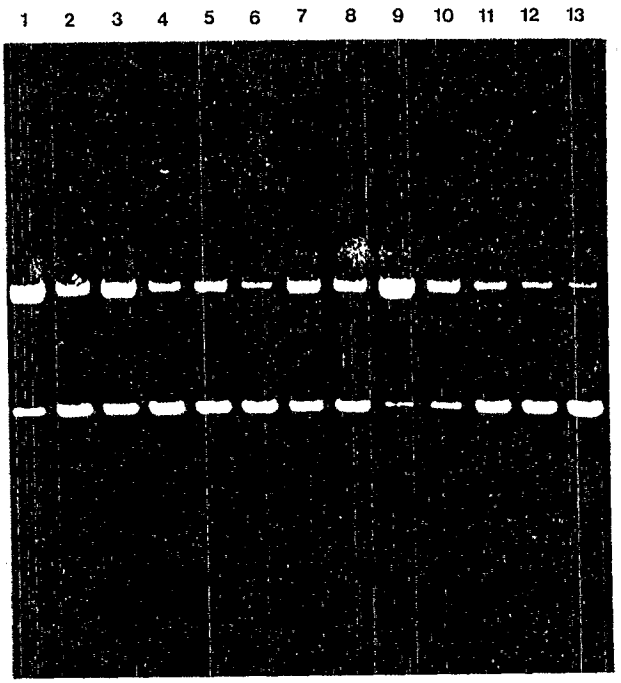


FIGURE 2

EFFECT OF VARIOUS PROCEDURES ON NUCLEASE ACTIVITY ASSOCIATED WITH DIFFERENT MICROSOMAL PREPARATIONS

Remarks:
- Lane 1: sucrose buffer, washed twice
- Lane 2: sucrose buffer and discontinuous metrizamide gradient
- Lane 3: phosphate buffer, washed twice
- Lane 4: phosphate buffer and discontinuous metrizamide gradient
- Lane 5: as Lane 4 (different preparation)
- Lane 6: phosphate buffer and 2 discontinuous metrizamide gradients
- Lane 7: phosphate buffer, washed twice with pyrophosphate
- Lane 8: "smooth" microsomes, from sucrose-cesium-chloride gradient
- Lane 9: "rough" microsomes, from sucrose-cesium-chloride gradient
- Lane 10: prepared according to previous INBIFO procedure
- Lane 11: as Lane 7 (different preparation)
- Lane 12: bicarbonate buffer (0.7 mol/l) followed by osmotic shock
- Lane 13: DNA control

All microsomal preparations were incubated with 2 ug DNA (20 mg/l) at a concentration of 0.1 g/l in the presence of EDTA (5 mmol/l) for 1 h.

DOCUMENTATION P 0500/5075 URO12RB15 7055 M

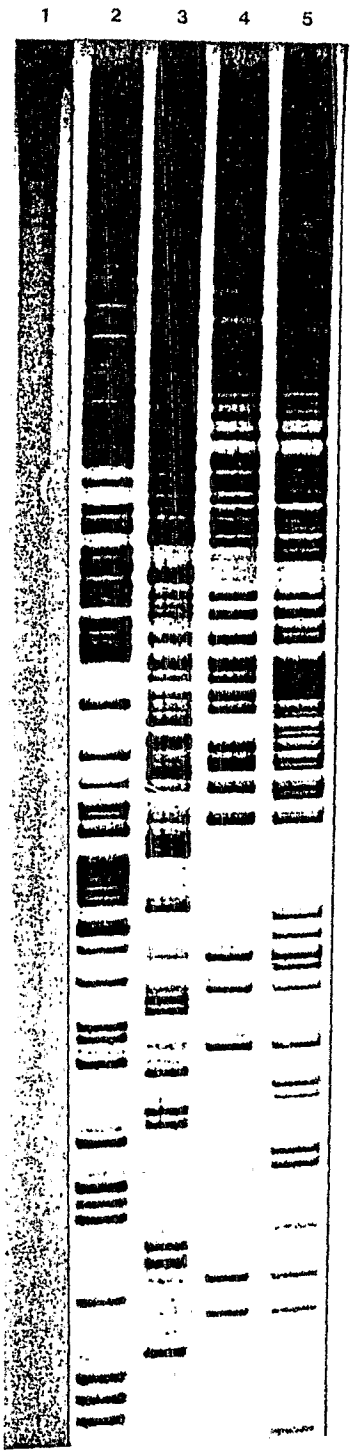


FIGURE 3

CONTROL SEQUENCES AND DNA NUCLEASE CONTROL OF pUR222 DNA

Remarks: Lane 1: DNA incubated with microsomes at 0.5 g microsomal protein/l for 1 h and loaded onto the gel without piperidine treatment

Lanes 2 to 5: control sequences G, A, C and C + T respectively

FIGURE 4

CONFORMATION ASSAY WITH BPDE

Remarks: Lane 1: BPDE at 0.01 mmol/l + 0.005 mmol NADPH/l

Lanes 2 to 5: BPDE at 0.01, 0.005, 0.001 and 0.0001 mmol/l respectively

Lane 6: control (untreated plasmid)

Incubation was for 30 minutes.

DOCUMENTATION P 0500/5075 URO12RB17 7042 M

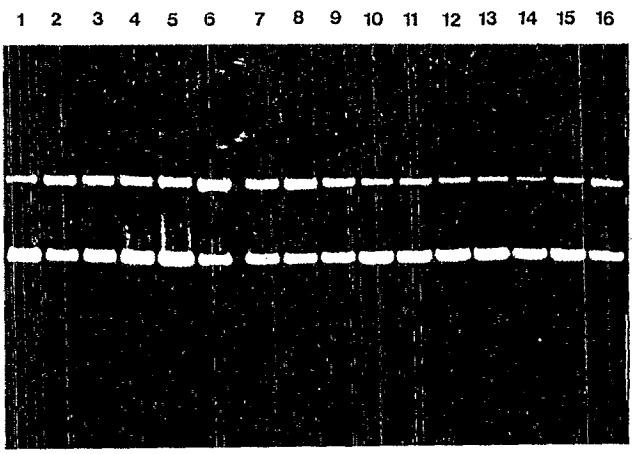


FIGURE 5A

CONFORMATION ASSAY WITH B(a)P AND CATECHOL

Remarks: Lane 1: control (plasmid incubated with microsomes)

Lane 2: B(a)P at 0.08 mmol/l with microsomal activation

Lanes 3 to 5: catechol at 1, 10 and 100 mmol/l respectively with microsomal activation

Lane 6: B(a)P at 0.008 mmol/l with microsomal activation

Lanes 7 to 9: B(a)P at 0.08 mmol/l plus catechol at 1, 10 and 100 mmol/l respectively (with microsomal activation)

Lanes 10 to 13: as 6 to 9 without microsomal activation

Lanes 14 to 16: as 3 to 5 without microsomal activation

Incubation was for 30 min.

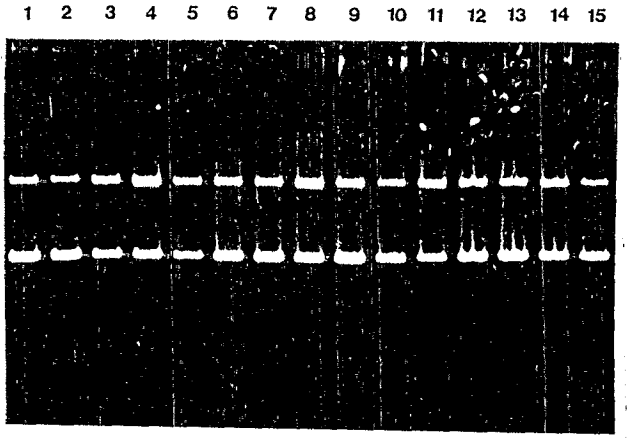


FIGURE 5B

CONFORMATION ASSAY WITH B(a)P AND CATECHOL

Remarks: Lanes 1 to 5: B(a)P at 0, 0.0001, 0.001, 0.01 and 0.1 mmol/l respectively

Lanes 6 to 10: catechol at 0, 0.1, 1, 10 and 100 mmol/l respectively

Lanes 11 to 15: B(a)P at 0.01 mmol/l plus catechol at 0, 0.1, 1, 10 and 100 mmol/l respectively.

Incubation was for 15 min. All samples contained microsomes.

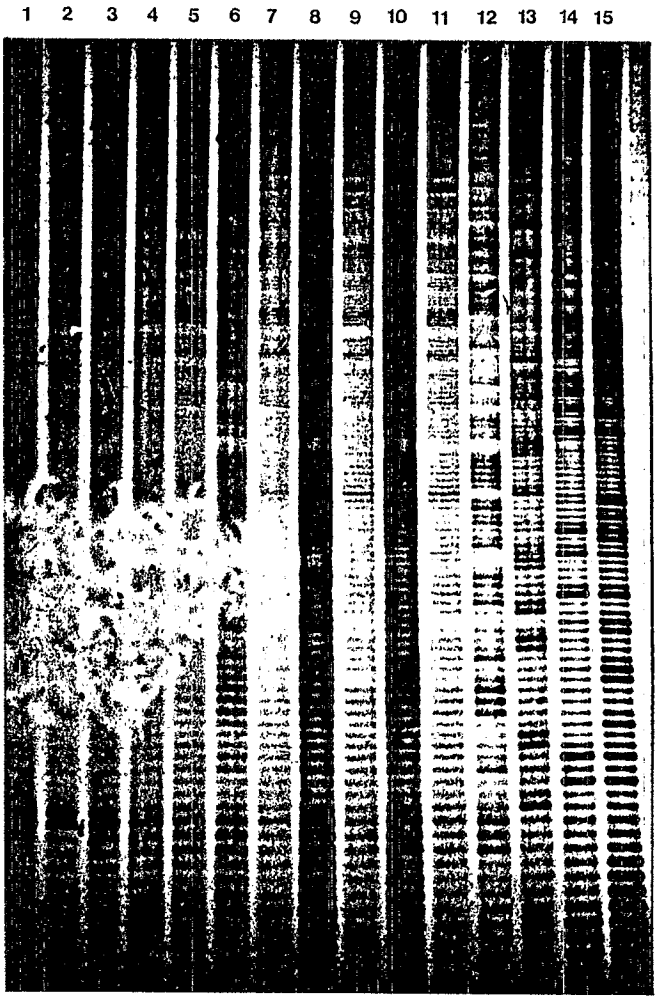


FIGURE 6

DNA ADDUCT FORMATION ON pUR222 WITH BPDE, CATECHOL AND FORMALDEHYDE

Remarks: Lanes 1 to 3: BPDE at 0.005, 0.0025 and 0.00125 mmol/l respectively.
Incubation was for 15 min.

Lanes 4 to 8: catechol at 10, 1, 0.1, 0.01 and 0.001 mmol/l respectively.
Incubation was for 1 h.

Lanes 9 to 11: formaldehyde at 1, 0.1 and 0.01 mmol/l respectively.
Incubation was for 1 h.

Lanes 12 to 15: control sequences G, A, C and C + T respectively

DOCUMENTATION P 0500/5075 URO12RB19 7042 M

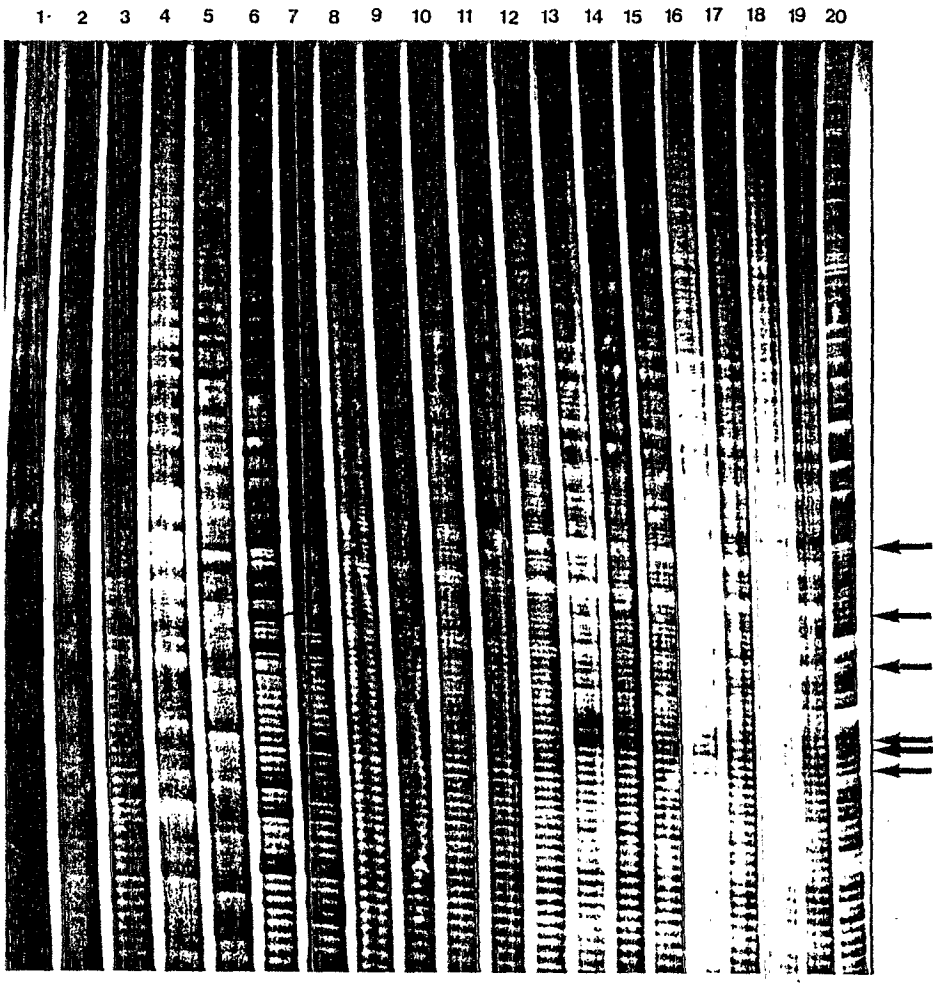


FIGURE 7

DNA ADDUCT FORMATION WITH BPDE, PYRENE AND CPP USING pUR222 AS TARGET DNA

Remarks: Lanes 1 to 3: BPDE, 0.1, 0.02 and 0.005 mmol/l respectively, 15 min incubation

Lanes 4 to 7: control sequences G, A, C, C + T respectively

Lanes 8 to 12: pyrene, 8, 10, 12: 1 mmol/l,
9, 11: 0.1 mmol/l,
8, 9: microsomes at 0.5 g/l,
10, 11: microsomes at 0.1 g/l,
12: no microsomes.

Incubation was for 1 h.

Lanes 13 to 19: CPP, 13 to 15 and 19: 10 mmol/l,
16 to 18: 1 mmol/l,
13, 16: microsomes at 2.5 g/l,
14, 17: microsomes at 0.5 g/l,
15, 18: microsomes at 0.1 g/l,
19: no microsomes.

Lane 20: control sequence G

Adducts are indicated by arrows.

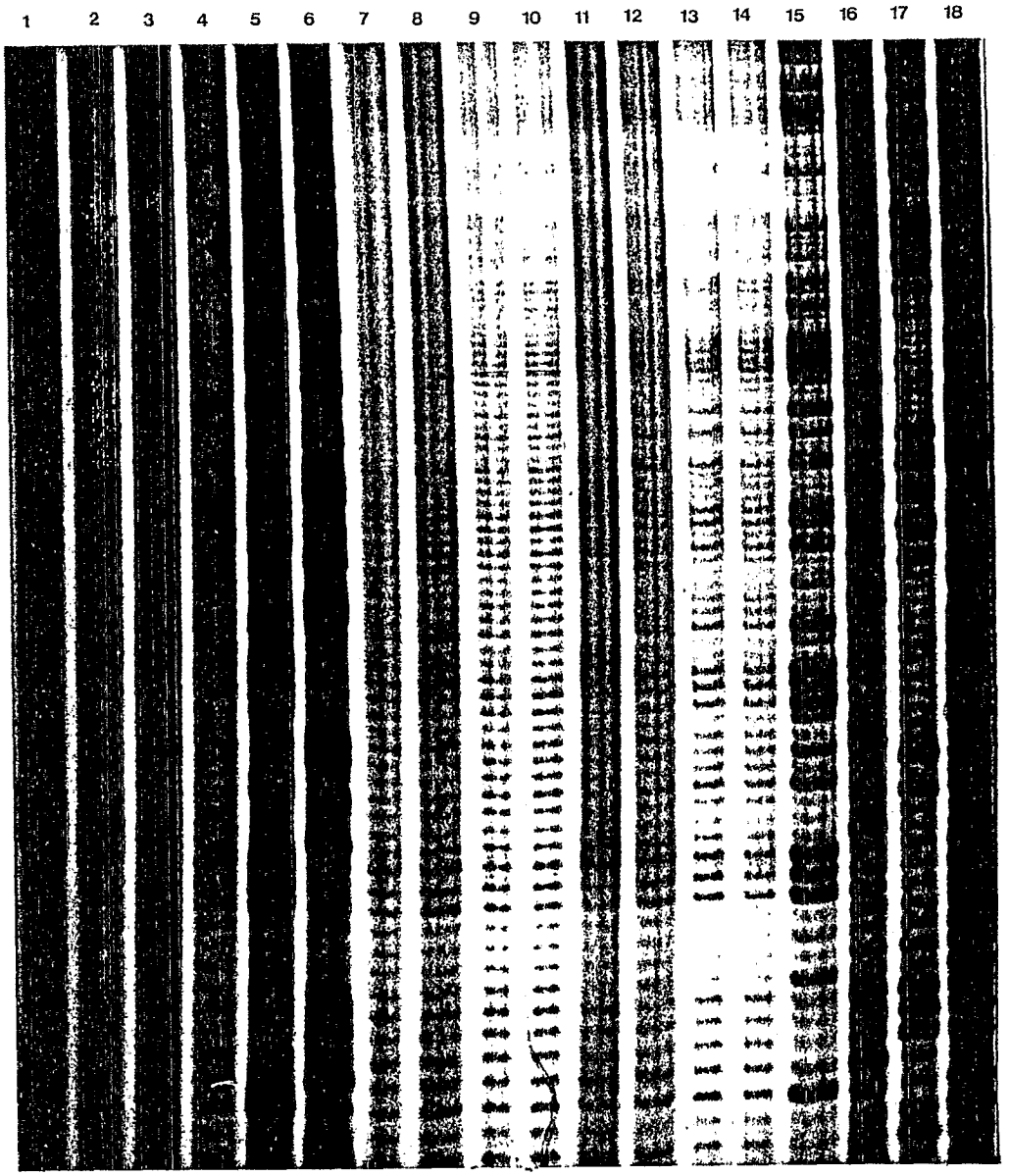


FIGURE 8

DNA ADDUCT FORMATION ON pUR222 WITH BPDE AND CATECHOL

Remarks: Lanes 1 to 3: BPDE at 0.005 mmol/l
Incubation was for 2, 15 and 60 min respectively.

Lane 4: acetone control

Lanes 5, 6: control sequences G, A

Lanes 7 to 14: BPDE at 0.005 mmol/l plus catechol
at the following concentrations:
7, 9, 11, 13: 1 mmol/l,
8, 10, 12, 14: 0.1 mmol/l,
7, 8, 11, 12: no microsomes,
9, 10, 13, 14: microsomes at 5 g/l
Incubation was for 15 min (7 to 10) and 1 h (11 to 14).

Lanes 15 to 18: control sequences G, A, C, C + T

DOCUMENTATION P 0500/5075 URO12RB22 7042 M

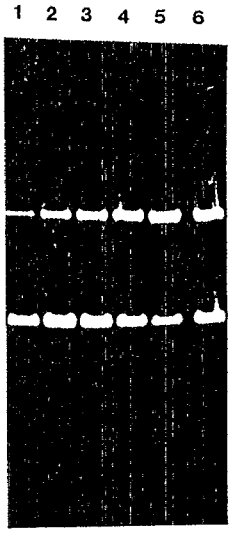


FIGURE 9

CONFORMATION ASSAY WITH DMBA

Remarks: Lanes 1 to 3: DMBA at 0, 1 and 5 mmol/l respectively with microsomal activation.
Incubation was for 15 min.

Lanes 4 to 6: as 1 to 3
Incubation was for 1 h.

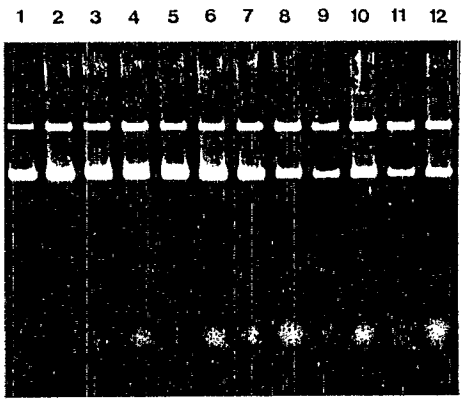


FIGURE 10

CONFORMATION ASSAY WITH PYRENE

Remarks: Lanes 1 to 6: pyrene at 0, 0.001, 0.01, 0.1, 1 and 5 mmol/l respectively with microsomal activation.
Incubation was for 15 min.

Lanes 7 to 12: as 1 to 6
Incubation was for 1 h.

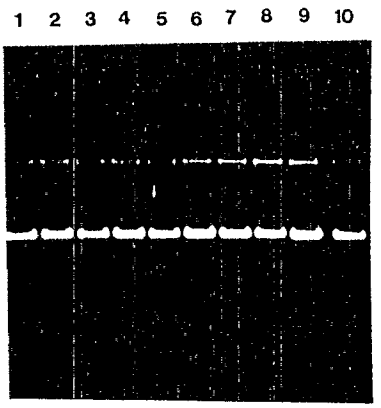


FIGURE 11

CONFORMATION ASSAY WITH CATECHOL WITHOUT MICROSOMAL ACTIVATION

Remarks: Lanes 1 to 5: catechol at 0, 0.1, 1, 10 and 100 mmol/l respectively.
Incubation was for 15 min.

Lanes 6 to 10: as 1 to 5.
Incubation was for 1 h.

DOCUMENTATION P 0500/5075 URO12RB25 7042 M

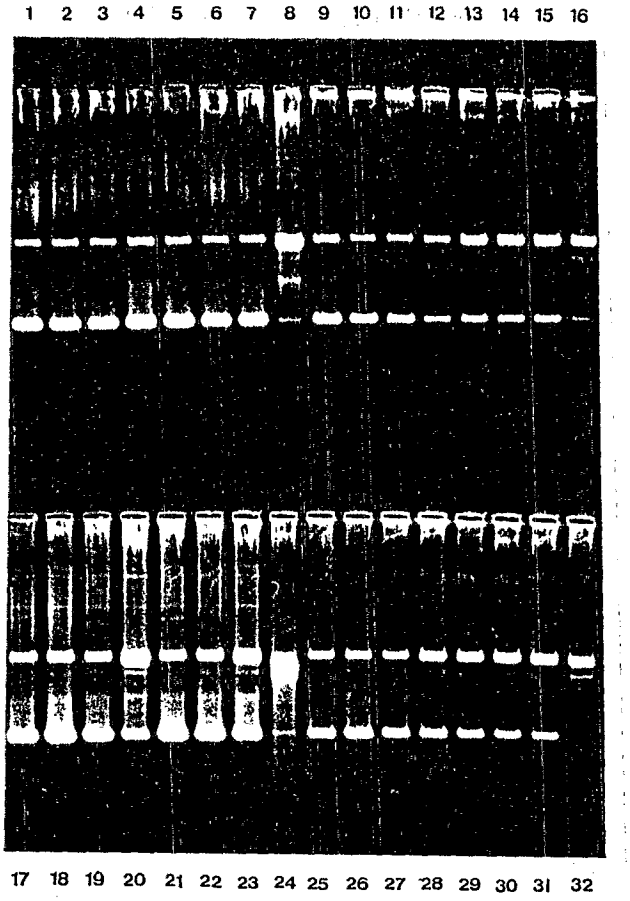


FIGURE 12

CONFORMATION ASSAY WITH CPP

Remarks: Lanes 1 to 4: CPP at 0, 0.1, 1 and 10 mmol/l respectively.
Incubation was for 15 min.

Lanes 5 to 8: as 1 to 4
Incubation was for 1 h.

Lanes 9 to 12: as 1 to 4 with the addition of microsomes

Lanes 13 to 16: as 5 to 8 with the addition of microsomes

Lanes 17 to 32: as 1 to 16 with heat posttreatment

DOCUMENTATION P 0500/5075 URO12RB26 7042 M

FIGURE 13

DNA ADDUCT FORMATION ON pUR222 WITH CPP

Remarks: Lanes 1 to 4: control sequences G, A, C and C + T respectively

Lanes 5 to 7: CPP at 10 mmol/l with microsomes at 2.5, 0.5 and 0 g/l respectively

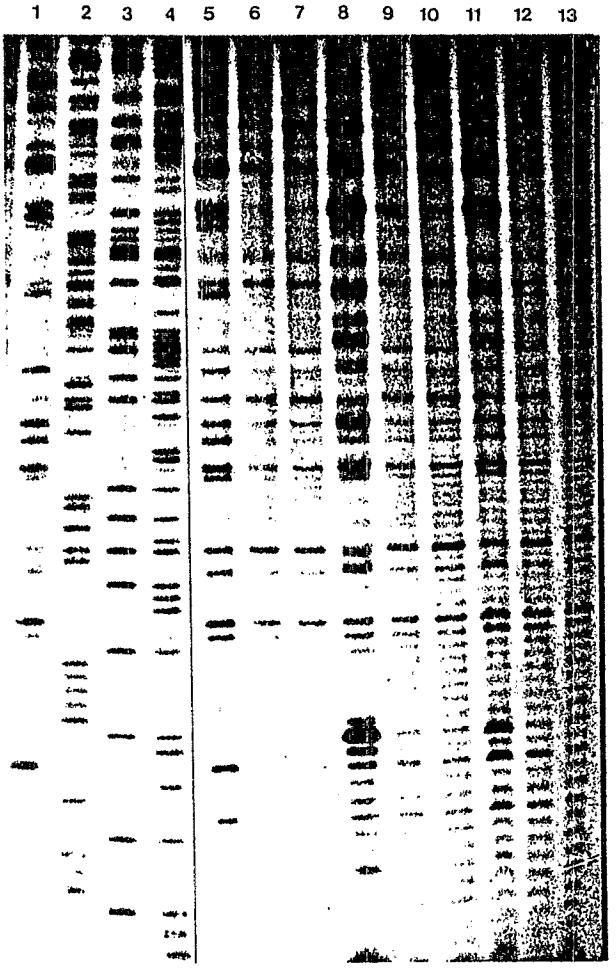


FIGURE 14

DNA ADDUCT FORMATION BY CPP USING THE PAL10 FRAGMENT AS TARGET

Remarks: Lanes 1 to 4: control sequences G, A, C and C + T respectively

Lanes 5 to 7: CPP, 10, 1 and 0.1 mmol/l respectively

Lanes 8 to 10: as 5 to 7, with microsomes at 0.5 g/l

Lanes 11 to 13: as 5 to 7, with microsomes at 2.5 g/l

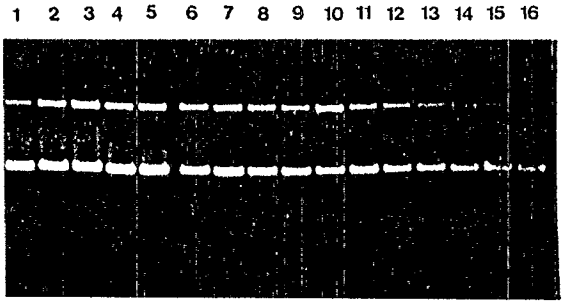


FIGURE 15

CONFORMATION ASSAY WITH DMNA

Remarks: Lanes 1 to 4: DMNA at 0.01, 0.1, 1 and 10 mmol/l respectively with microsomes at 0.1 g/l

Lanes 5 to 8: as 1 to 5 with microsomes at 0.5 g/l

Lanes 9, 10: nuclease controls, microsomes at 0.1 and 0.5 g/l respectively

Lanes 11, 12: as 9, 10 without NADPH

Lanes 13 to 16: as 1 to 4 without NADPH

Incubation was for 15 min.

DOCUMENTATION P 0500/5075 URO14RA7 7070 M

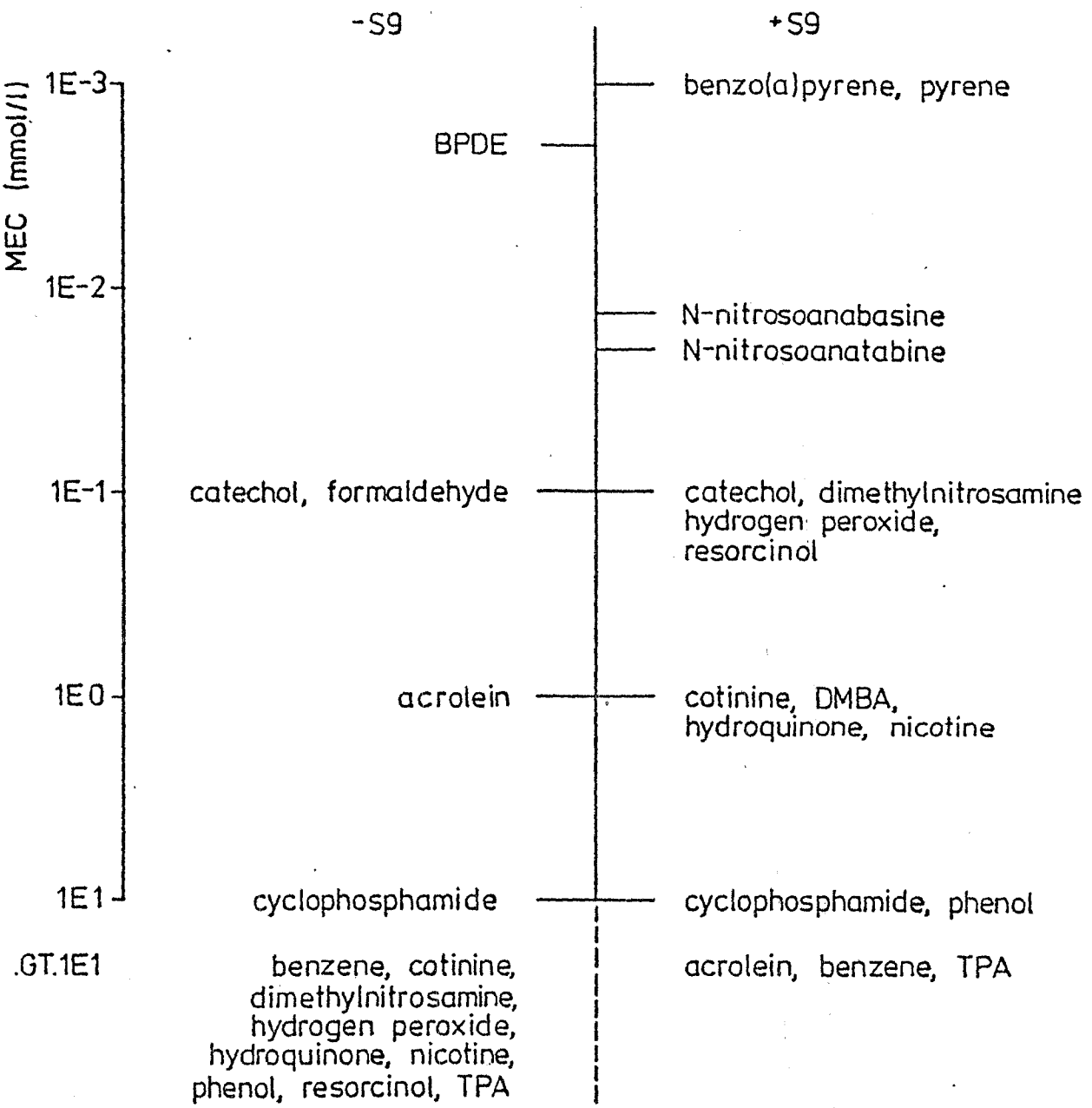


FIGURE 16

RESULTS OF THE IN VITRO DNA ADDUCT FORMATION ASSAY

Remarks: Due to their low solubility in the solvents used the following substances were assayed but did not show any effect up to concentrations:

-S9: .GT.5E-2 mmol/l: benzanthracene, N-nitrosonornicotine, N-nitrosoanabasine N-nitrosanatabine, 4-N-methyl--N-nitrosoamino-1,3-pyridy butanone

.GT.1E-1 mmol/l: benzo(a)pyrene

.GT.5 mmol/l: DMBA, pyrene

+S9: .GT.5E-2 mmol/l: benzanthracene, N-nitrosonornicotine, 4-N-methyl--N-nitrosoamino-1,3-pyridyl-butanone

7 REFERENCES

Atcheson, M., Chu, C., Kakunaga, T., Van Duuren, B.L., Chemical cocarcinogenesis with the use of a subclone derived from Balb/3T3 cells with catechol as cocarcinogen, J. Natl. Cancer Inst. 69 : 503-508 (1982)

Auerbach, C., Moutschen-Dahmen, M., Moutschen, J., Genetic and cytogenetical effects of formaldehyde and related compounds, Mutat. Res. 39 : 317-362 (1977)

Baturay, N., Kennedy, A.R., Pyrene acts as a carcinogen with the carcinogens benzo(a)pyrene, beta-propiolactone and radiation in the induction of malignant transformation in cultured mouse fibroblasts; soybean extract containing the Bowman-Birk inhibitor acts as an anticarcinogen, Cell Biol. Toxicol. 2 : 21-32 (1986)

Bichara, M., Fuchs, P.P., DNA binding and mutation spectra of the carcinogen N-2-aminofluorene in Escherichia coli. A correlation between the conformation of the premutagenic lesion and the mutation specificity, J. Molec. Biol. 183 : 341-351 (1985)

Bigger, C.A.H., Dipple, A., Carcinogen-DNA binding: a probe for metabolic activation in vivo and in vitro, in: Finley, T.W., Schwarz, D.E. (Eds.): Xenobiotic metabolism: nutritional effects, American Chemical Society Symposium Series No. 277, American Chemical Society, 1985, pp. 187-208

Bina-Stein, M., Tritton, T.R., Aurintricarboxylic acid is a nonspecific enzyme inhibitor, Mol. Pharmacol. 12 : 191-193 (1976)

Blumenthal, T., Landers, T.A., The inhibition of nucleic acid-binding proteins by aurintricarboxylic acid, Biochem. Biophys. Res. Commun. 55 : 680-688 (1973)

Boiteux, S., Laval, J., Coding properties of poly(deoxycytidylic acid) templates containing uracil or apyrimidinic sites: in vitro modulation of mutagenesis by deoxyribonucleic acid repair enzymes, Biochemistry 21 : 6746-6751 (1982)

Boles, T.C., Hogan, M.E., Site-specific carcinogen binding to DNA, Proc. Natl. Acad. Sci. USA 81 : 5623-5627 (1984)

Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heynecker, H.L., Boyer, H.W., Crosa, J.H., Falkow, S., Construction and characterisation of new cloning vehicles. II. A multipurpose cloning system, Gene 2 : 95-113 (1977)

Borish, E.T., Cosgrove, J.P., Church, D.F., Deutsch, W.A., Pryor, W.A., Cigarette tar causes single-strand breaks in DNA, Biochem. Biophys. Res. Comm. 133 : 780-786 (1985)

Bradley, M.O., Bhuyan, B., Francis, M.C., Langenbach, R., Peterson, A., Huberman, E., Mutagenesis by chemical agents in V79 Chinese hamster cells: a review and analysis of the literature, Mut. Res. 87 : 81-142 (1981)

Brown, B.R., Firth, W.J., Yielding, L.W., Acridine structure correlated with mutagenic activity in Salmonella, Mutat. Res. 72 : 373-388 (1980)

Bruce, W.R., Heddle, J.A., The mutagenic activity of 61 agents as determined by the micronucleus Salmonella, and sperm abnormality assays, Can. J. Genet. Cytol. 21 : 319-334 (1979)

Bull, J.M.C., An update on the anticancer effects of a combination of chemotherapy and hyperthermia, Cancer Res. 44 : 4853-4856 (1984)

Cooper, C.S., Gerwin, B.I., Scheiner, L.A., Sites of single-strand breaks in DNA treated with a diol-epoxide of benz(a)anthracene, Carcinogenesis 4 : 1645-1649 (1983)

Di Giovanni, J., Fisher, E.P., Sawyer, T.W., Kinetics of formation and disappearance of 7,12-dimethylbenz(a)anthracene: DNA adducts in mouse epidermis, Cancer Res. 46 : 4400-4405 (1986)

Eisenstadt, E., Warren, A.J., Porter, J., Atkins, D., Miller, J.H., Carcinogenic epoxides of benzo(a)pyrene and cyclopenta(cd)-pyrene induce base substitutions via specific transversions, Proc. Natl. Acad. Sci. USA 79 : 1945-1949 (1982)

El-Bayoumy, K., Hecht, S., Mutagenicity of K-region derivatives of 1-nitropyrene: remarkable activity of 1- and 3-nitro-5-H-phenanthro (4,5-bcd)pyran-5-one, Mutat. Res. 170 : 31-40 (1986)

Fahl, E.W., Lalwani, N.D., Watanabe T., Goel, S.K., Reddy, J.K., DNA damage related to increased hydrogen peroxide generation by hypolipidemic drug-induced liver peroxisomes, Proc. Natl. Acad. Sci. USA 81 : 7827-7830 (1984)

Federal Register, USA, Nonclinical laboratory studies: good laboratory practice regulations, 43 (248) : 59986-60025 (1978)

Ferguson, L.R., Denny, W.A., Mc Phee, D.G., Three consistent patterns of response to substituted acridines in a variety of bacterial tester strains used for mutagenicity testing, Mutat. Res. 157 : 29-37 (1985)

Frazelle, T.H., Abernethy, D.J., Boreiko, C.T., Weak promotion of C3H/10T1/2 cell transformation by repeated treatments with formaldehyde, Cancer Res. 43 : 3236-3239 (1983)

Gelboin, H.V., Benzo(a)pyrene metabolism, activation and carcinogenesis: role and regulation of mixed-function oxidases and related enzymes, Physiol. Rev. 60 : 1107-1166 (1980)

Goldmacher, V.S., Thilly, W.G., Formaldehyde is mutagenic for cultured human cells, Mut. Res. 116 : 417-422 (1983)

Goth, R., Rajewsky, M.F., Molecular and cellular mechanisms associated with pulse-carcinogenesis in the rat nervous system by ethylnitrosourea: ethylation of nucleic acids and elimination rates of ethylated bases from the DNA of different toxins, Z. Krebsforsch. 82 : 37-64 (1974)

Grafstrom, R.C., Fornace, A., Harris, C.C., Repair of DNA damage caused by formaldehyde in human cells, Cancer Res. 44 : 4323-4327 (1984)

Grover, P.L. (Ed.): Chemical carcinogens and DNA, Vol. I, Boca Raton: CRC Press, 1979

Haar, H., Marshall, G.J., Bierman, H.R., Steinfeld, J.L., The influence of Cyclophosphamide upon neoplastic diseases in man, Cancer Chemother. Rep. 6 : 41-51 (1960)

Hales, B.F., Modification of the mutagenicity and teratogenicity of cyclophosphamide in rats with inducers of the cytochromes P-450, Teratology 24: 1-11 (1981)

Harris, C.C., Vahakangas, K., Newman, M.J., Trivers, G.E., Shamsuddin, A., Sinopoli, N., Mann, D.L., Wright, W.E., Detection of benzo(a)pyrene diol epoxide-DNA adducts in peripheral blood lymphocytes and antibodies to the adducts in serum from coke oven workers, Proc. Natl. Acad. Sci. USA 82 : 6672-6676 (1985)

Hecht, S.S., Cornella, S., Mori, H., Hoffmann, D., A study of tobacco carcinogenesis. XX. Role of catechol as a major cocarcinogen in the weakly acid fraction of smoke condensate, J. Nat. Cancer Inst. 66 : 163-169 (1981)

Heck, H.D., Casanova-Schmitz, M., Reactions of formaldehyde in the rat nasal mucosa, Formaldehyde: Toxicol. Epidemiol. Mech. Pap. Meet. J. 2nd : 211-223 (1983)

Heidelberger, C., Chemical carcinogenesis, Ann. Rev. Biochem. 44 : 79-121 (1975)

Hermann, M., Synergistic effects of individual polycyclic aromatic hydrocarbons in the mutagenicity of their mixtures, Mutat. Res. 90 : 399-409 (1981)

Hoffmann, D., Melikian, A., Adams, J.D., Brunnemann, K.D., Haley, N.J., New aspects of tobacco carcinogenesis, Carcinogenesis 8 : 239-256 (1985)

Hsu, I.C., Cole, K., Lipsky, M., Trump, B.F., Responses of cultured hepatocytes to chemical carcinogens, Proc. Annu. Meeting Am. Assoc. Cancer Res. 27 : 79 (1986)

Huberman, E., Sachs, L., Mutability of different genetic loci in mammalian cells by metabolically activated carcinogenic polycyclic hydrocarbons, Proc. Natl. Acad. Sci. USA 73 : 188-192 (1976)

Hunstein, W., Tumor inductions by cytostatics in man, Recent Results Cancer Res. 52 : 50 (1975)

IARC Working Group, IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans, Vols. 1-26, Lyon: International Agency for Research on Cancer, 1972-1982

INBIFO study P 0500/3060,
Establishment of an in vitro DNA adduct formation assay or direct acting mutagens (PT),
Study director: Dr.rer.nat. R.-A. Walk
not yet reported

Inouye, S., Site-specific cleavage of double-strand DNA by hydroperoxide of linoleic acid, FEBS letters 172 : 231-234 (1984)

International Agency for Research on cancer: Monographs on the evaluation of the carcinogenic risk of chemicals to humans, Vol. 32, Lyon, France: World Health Organisation, 1983, pp. 211-224

Jeffrey, A.M., DNA modification by chemical carcinogens, Pharmacol. Ther. 28 : 237-272 (1985)

Jeffrey, A.M., Grzeskowick, K., Weinstein, I.B., Nakanishi, K., Roller, P., Harvey, R.G., Benzo(a)pyrene-7,8-dihydrodiol-9,10-oxide adenosine and deoxyadenosine adducts: structure and stereochemistry, Science 206 : 1309 (1979)

Jeffrey, A.M., Kinoshitz, T., Santella, R.M., Grauberger, D., Katz, L., Weinstein, I.B.: The chemistry of polycyclic aromatic hydrocarbon-DNA adducts, in: Pullman, B., Tso, P.O.P., Gelboin, H. (Eds.): Carcinogenesis: Fundamental mechanisms and environmental effects, New York: Academic Press, 1980, pp. 505-579

Kerns, W.D., Pavkov, K.L., Donofrio, D.J., Gralla, E.J., Swenberg, J.A., Carcinogenicity of formaldehyde in rats and mice after long-term inhalation exposure, Cancer Res. 43 : 4382-4392 (1983)

Kier, L.E., Brusik, D.J., Auletta, A.E., von Halle, E.S., Brown, M.M., Simmon, V.J., Dunkel, V., McCann, J., Mortelmans, K., Prival, M., Rao, T.K., Ray, V., The Salmonella typhimurium/ mammalian microsomal assay, a Report of the U.S. Environmental Protective Agency Gen-Tox Program, Mutat. Res. 168 : 69-240 (1986)

Kircher, M., Fleer, R., Ruhland, A., Brendel, M., Biological and chemical effects of mustard gas in yeast, Mutat. Res. 63 : 273-289 (1979)

Kohn, K.W., Ewig, R.A.G., Erickson, L.C., Zwelling, L.A., Measurement of strand breaks and crosslinks of alkaline elution, in: Friedberg, E. C., Hanawalt, P.C. (Eds.): DNA Repair: A Laboratory Manual of Research Procedures, New York: Marcel Dekker, 1981, pp. 379-401

Kornbrust, D., Dietz, D., Aroclor-1254 pretreatment effects on DNA-repair in rat hepatocytes elicited by in vivo or in vitro exposure to various chemicals, Environ. Mutagen. 7 : 857-870 (1985)

Kreiger, R.A., Garry, V.F., Formaldehyde-induced cytotoxicity and sister chromatid exchanges in lymphocyte cultures, Mutat. Res. 120 : 41-55 (1983)

Lane, M., Some effects of cyclophosphamide (cytoxan) on normal mice and mice with L1210 leukemia, J. Natl. Cancer Inst. 23 : 1347-1359 (1959)

Levy, S., Noncentini, S., Billardon, C., Induction of cytogenic effects in human fibroblast cultures of the exposure to formaldehyde or ex-rays, Mutat. Res. 119 : 309-317 (1983)

Lohman, P.H.M., Baan, R.A., Fichtinger-Shepman, A.M.J., Muysken-Schoen, M.A., Lansbergen, R.J., Berends F., Molecular dosimetry of genotoxic damage: biochemical and immunochemical methods to detect DNA damage, Trends in Pharmacol. Sci. Nov., 5-11 (1985)

Loquet, C., Engelhardt, U., Schaefer-Ridder, M., Differentiated genotoxic response of carcinogenic and non-carcinogenic benzacridines and metabolites in rat hepatoma cells, Carcinogenesis 6 : 455-457 (1985)

Lu, L.-J.W., Disher, R.M., Reddy, M.V., Randerath, K., 32P-postlabeling assay in mice of transplacental DNA damage induced by the environmental carcinogens safrole, 4-aminobiphenyl and benzo-(a)pyrene, Cancer Res. 46 : 3046-3054 (1986)

Maniatis, T., Fritsch, E.F., Sambrook, J. (Eds.): Molecular Cloning, A Laboratory Manual, New York: Cold Spring Habor Laboratory, 1982, pp. 104-106, p. 113 ff, p. 173 ff, p. 475 ff

Mansfield, C.T., Hodge, B.T., Hege, R.B., Hamlin, W.C., Analysis of formaldehyde in tobacco smoke by high performance liquid chromatography, J. Chrom. Sci. 15 : 301-302 (1977)

Marks, F., Hecker, E., Metabolism and mechanism of action of oestrogens. XII. Structure and mechanism of formation of water-soluble and protein-bound metabolites of oestrone in rat liver microsomes in vitro and in vivo, Biochim. Biophys. Acta 187 : 250-265 (1969)

Maxam, A.M., Gilbert, W.: Sequencing end-labeled DNA with base-specific chemical cleavages, in: Grossman, L., Moldave, K. (Eds.): Methods in enzymology, Vol. 65, New York: Academic Press, 1980, pp. 499-560

McCann, J., Choi, E., Yamasaki, E., Ames, B.N., Detection of carcinogens as mutagens in the Salmonella/microsome test, Proc. Natl. Acad. Sci. (USA) 72 : 5135-5139 (1975)

Melikian A.A., Leszczynska, J.M., Hecht, S.S., Hoffmann, D., Effects of the co-carcinogen catechol on benzo(a)pyrene metabolism and DNA adduct formation in mouse skin, Carcinogenesis 7 : 9-15 (1986) - (INBIFO reference: L694/E13)

Mirvish, S.S., Salmasi, S.H., Lawson, T.B., Pour, P., Sutherland, D., Test of catechol, tannic acid, bidens pilosa, croton oil and phorbol for cocarcinogenesis of oesophogeal tumours induced in rats by methyl-n-amyl-nitrosamine, J. Natl. Cancer Inst. 74 : 1238-1289 (1985)

Morimoto, K., Wolff, S., Increase of sister chromatid exchanges and perturbations of cell division kinetics in human lymphocytes by benzene metabolites, Cancer Res. 40 : 1189-1193 (1980)

Natarajan, A.T., Darroudi, F., Bussman, C.J.M., van Kesteren-van Leeuwen, A.C., Evaluation of the mutagenicity of formaldehyde in mammalian cytogenetic assays in vivo and in vitro, Mutat. Res. 122 : 355-360 (1983)

Osborne, M.R., Jacobs, S., Harvey, R.G., Brookes, P., Minor products from the reaction of (+) and (-) benzo(a)pyrene-anti-diolepoxides with DNA, Carcinogenesis 2 : 553-558 (1981)

Pegg, A.E., Metabolism of N-nitrosodimethylamine, in: Montesano, R., Bartsch, H., Tomatis, L. (Eds.): Molecular and cellular aspects of carcinogen screening tests, Lyon, France: IARC Scientific Publication No. 27, International Agency for Research on Cancer, 1980, pp. 3-22

Perera, F.P., Poirier, M.C., Yuspa, S.H., Nahayama, J., Jaretzki, A., Curnen, M.M., Knowles, D.M., Weinstein, I.B., A pilot project in molecular cancer epidemiology: determination of benzo(a)pyrene-DNA adducts in animal and human tissues by immunoassays, Carcinogenesis 3 : 1405-1410 (1982)

Perin-Roussel, O., Barat, N., Zajdela, F., Formation and removal of dibenzo(a,e)fluoranthene-DNA adducts in mouse embryo fibroblasts, Carcinogenesis 6 : 1791-1796 (1985)

Prusik, T., Spectroscopic methods for the investigation of the structures of benzo(a)pyrene-DNA adducts, Diss. Abstr. Int. (B) : 42 : 649-B (1981)

Rahn, R.O., Chang, S.S., Holland, J.M., Shugart, L.R., A fluorometric-HPLC assay for quantitating the binding of benzo(a)pyrene metabolites to DNA, Bioch. Biophys. Res. Comm. 109 : 262-268 (1982)

Reddy, M.V., Randerath, K., Nuclease P1-mediated enhancement of sensitivity of 32P-postlabeling test for structurally diverse DNA adducts, Carcinogenesis 7 : 1543-1551 (1986)

Reich, E., Franklin, R.M., Shatkin A.J., Tatum, E.L., Action of actinomycin D on animal cells and viruses, Proc. Natl. Acad. Sci. USA 48 : 1238-1245 (1962)

Report of the Federal Panel on Formaldehyde, Environ. Health Perspect. 43 : 139-168 (1982)

Rice, J.E., Hosted, T.J., Lavoie, E.J., Fluoranthene and pyrene enhance benzo(a)pyrene-DNA adduct formation in vivo in mouse skin, Cancer Lett. 24 : 327-333 (1984) - (INBIFO reference: L654/E8)

Rinkus, S.J., Legator, M.S., Chemical characterisation of 465 known or suspected carcinogens and their correlation with mutagenic activity in the Salmonella typhimurium system, Cancer Res. 39 : 3289-3318 (1979)

Rüther, U., Koenen, M., Otto, K., Müller-Hill, B., pUR222, a vector for cloning and rapid chemical sequencing of DNA, Nucl. Acids Res. 9 : 4087-4098 (1981)

Sage, E., Haseltine, W.A., High ratio of alkali-sensitive lesions to total DNA modification induced by benzo(a)pyrene diol epoxide, J. Biol. Chem. 259 : 11098-11102 (1984)

Sawadaishi, K., Miura, K., Ohtsuha, E., Ueda, T., Ishizaki, K., Shinriki, N., Ozonolysis of supercoiled pBR322 DNA resulting in strand cission to open circular DNA, Nucleic Acids Res. 13 : 7183-7194 (1985)

Schaaper, R.M., Kunkel, T.A., Loeb, L.A., Proc. Natl. Acad. Sci. USA 80 : 487-491 (1982)

Schauenstein, E., Esterbauer, H., Formation and properties of reactive aldehydes, in: Submolecular biology and cancer, Ciba Foundation Series 67, Excerpta Medica, pp. 225-241 (1978)

Schechtman, L.M., Gallagher, M.A., Henry, C.J., Kouri, R.E., Lubet, R.A., Induction of neoplastic transformation and single strand DNA breaks in C 10T1/2 cells by polycyclic hydrocarbons and short chain alkylating agent, Fed. Proc. 41 : 379 (1982)

Sengupta, S.K., Evaluation of new chromophore modified actinomycin D (mice, rats), Crisp Data Base National Inst. of Health, Boston, University, Massachusetts

Sengupta, S.K., Blondin, J., Szabo, J., Covalent binding of isomeric 7-(2.3-epoxypropoxy)actinomycin D to DNA, J. Med. Chem. 27 : 1465-1470 (1984)

Sieber, S.M., Adamson, R.H., Toxicity of antineoplastic agents in man, chromosomal aberrations, antifertility effects, congenital malformations, and carcinogenic potential, Adv. Cancer Res. 22 : 57-155 (1975)

Sims, P., The metabolic activation of chemical carcinogens, Brit. Med. Bulletin 33 : 11-18 (1980)

Sims, P., Grover, P.L.: Involvement of dihydrodiols and diol epoxides in the metabolic activation of polycyclic hydrocarbons other than benzo(a)pyrene, in: Gelboin, H.V., Ts'o, P.O.P. (Eds.): Polycyclic hydrocarbons and cancer, Vol. 3, New York: Academic Press, 1981, pp. 117-181

Sina, J.F., Bean, C.L., Dysart, G.R., Taylor, V.I., Bradley, M.O., Evaluation of the alkaline elution/rat hepatocyte assay as a predictor of carcinogenic/mutagenic potential, Mutat. Res. 113 : 357-391 (1983) - (INBIFO reference: L657/D14)

Singer, B., N-nitrosoalkylating agents: formation and persistence of alkyl derivatives in mammalian nucleic acids as contributing factors in carcinogenesis, J. Natl. Cancer Inst. 62 : 1329-1339 (1979)

Singer, B., Grunberger, D. (Eds.): Molecular biology of mutagens and carcinogens, New York and London: Plenum Press, 1983

Skare, J., Schrotel, K.R., Alkaline elution of rat testicular DNA: detection of DNA cross-links after in vivo treatment with chemical mutagens, Mutat. Res. 130 : 295-303 (1984)

Struck, R.F., Kirk, M.C., Mellett, L.B., El Dareen, S., Hill, D.L., Urinary metabolites of the antitumor agent cyclophosphamide, Molec. Pharmacol. 7 : 519-529 (1971)

Sutcliffe, J.G., Cold Spring Harbour, Lab. Symposia 43 : 77 (1979)

Swenberg, J.A., Kerns, W.D., Mitchell, R.I., Gralla, E.T., Pavkov, K.L., Induction of squamous cell carcinomas of the rat nasal cavity by inhalation exposure to formaldehyde vapour, Cancer Res. 40 : 3398-3402 (1980)

Van Cantfort, J., De Graeve, J., Gielen, J.E., Radioactive assay for aryl hydrocarbon hydroxylase: Improved method and biological importance, Biochem. Biophys. Res. Commun. 79 : 505-512 (1977)

Van Duuren, G.L., Goldschmidt, B.M., Cocarcinogenic and tumor-promoting agents in tobacco carcinogenesis, J. Natl. Cancer Inst. 56 : 1237-1242 (1976)

Wakata, A., Oka, N., Hiramoto, K., Yoshioka, A., Negishi, K., Wataya, Y., Hayatsu, H., DNA strand cleavage in vitro by 3-hydroxyamino-1-methyl-5H-pyrido(4,3-b)indole, a direct-acting mutagen formed in the metabolism of carcinogenic 3-amino-1-methyl-5H-pyrido (4,3-b)indole, Cancer Res. 45 : 5867-5871 (1985)

Weichselbaum, C.T.E., An accurate and rapid method for the determination of proteins in small amounts in blood serum and plasma, Am. J. Clin. Pathol. 16 : 40-49 (1946)

Witkin, E.M., Ultarviolet mutagenesis and inducible DNA repair in Escherichia coli, Bacteriol. Rev. 40 : 869-907 (1976)

Williams, G.M., Laspia, M.F., Dunkel, V.C., Reliability of the hepatocyte primary culture/DNA repair test in testing of coded carcinogens and noncarcinogens, Mutat. Res. 97 : 359-370 (1982)

Wynder, E.L., Hoffmann, D.C., Tobacco and health: a societal challenge, New Engl. J. Med. 300 : 894-903 (1979)

Yamada, K., Shirahata, S., Murakamii, H., Nishiyama, K., Shinohara, K., Omura, H., DNA breakage by phenyl compounds, Agric. Biol. Chem. 49 : 1423-1428 (1985)

Yoshida, D., Fukuhara, Y., Mutagenicity and co-mutagenicity of catechol on Salmonella, Mutat. Res. 120 : 7-11 (1983) - (INBIFO reference: L67/G7)

END OF DOCUMENTATION